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The Journal of Biochemistry, Vol. 37, No. 2.

# THE CHANGE OF N-SUBSTITUTED AMINO ACID IN THE ANIMAL BODY

## XIII. THE FATE OF DL-α-N-METHYLHISTIDINE IN THE ANIMAL BODY AND ITS ENZYMATIC DECOMPOSITION

By

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(Received for publication January 23, 1950)

In spite of the many valuable works concerning the metabolism of histidine, such as those of Kotake and Konishi (1922), Konishi (1922, 1925), Kiyokawa (1932), Kaufmann and Mislowitzer (1930, 1931), Edlbacher and his coworkers (1926, 1930-31, 1934), Abderhalden *et al.* (1931, 1937), Sera and Yada (1939, 1940), and Takeuchi (1941), little is decided about its intermediary metabolism in the animal body. Because, the experiments of Edlbacher, Abderhalden, Kaufmann, Sera and Takeuchi are pure enzymic ones, the dogs of both Kotake and Jaffe are considered by some authors as particular cases, and Darby *et al.* could obtaine no urocanic acid in the urine of rabbits following the parenteral administration of L-histidine, and concluded that urocanic acid was not a quantitatively important intermediate in the normal metabolism of histidine.

The following article may give some suggestions on the intermediary metabolism of histidine. DL-α-N-methylhistidine was administered to a dog subcutaneously, imidazolepyruvic acid was isolated from its urine as dinitrophenylhydrazone together with unchanged DL-α-N-methylhistidine. According to the investigations of Hirohata and his coworkers, demethylase oxidizes L-α-N-methyl-

amino acids and splits them into formaldehyde and corresponding amino acids. Therefore DL-α-N-methylhistidine will be partly decomposed into L-histidine in the body and should become urocanic acid as Kotake and his coworkers assert. Notwithstanding my efforts, however, all attempt of isolation of urocanic acid failed, although I confirmed previously the formation of urocanic acid from L-histidine on the same dog in the case of parenteral administration.

In an enzymic experiment in vitro, kidney and liver extracts of rabbits were reacted with DL-α-N-methylhistidine succesivly, and imidazolepyruvic acid was a'so isolated together with urocanic acid.

## Experimental

1. As a preliminary experiment a neutralized solution of 5 g. L-histidine monohydrochloride was administered subcutaneously to 2 dogs. The collected urine of each dog for 4 days was evaporated on a water bath to dryness and extracted repeatedly with ethanol until the residue no longer showed Pauly's reaction. After distilling off the alcohol *in vacuo*, a small amount of water was added, the solution was treated with norit, sulphuric acid added in 5 % concentration, and phosphotungstic acid. It was allowed to stand over night, filtered by suction, decomposed with baryta, the excess of baryta was removed with sulphuric acid quantitatively, and the filtrate from the barium sulphate was concentrated under diminished pressure. Picric acid solution, was added and the sample kept in the ice box for a week. 0.4 g. and 0.034 g. urocanic acid picrate respectively were obtained. Both melted at 194°.

N determination: $C_{12}H_9O_9N_5$ calculated N 19.07 %
found 18.08 %

2. DL-α-N-Methylhistidine hydrochloride was prepared according to the method of Gerngross, Fargher and Pyman. It melted at 133°–134°.

N determination: $C_9H_{11}O_2N_3 \cdot 2HCl + H_2O$ calculated N 16.16 %
found N 15.60 %

6.8 g. of DL α-N-methylhistidine was injected in the dog of the first experiment, the combined urine (for 4 days) was treated as above, but mercuric chloride was added instead of picric acid. The resulting precipitate was filtered, suspended in water, treated with hydrogen sulfide. The filtrate from mercuric sulfide was evaporated *in vacuo*, the residue was moisted with water and evaporated under reduced pressure. This treatment was repeated three times to remove all hydrogen chloride. A small amount of water was added to the residue, it was acidified with sulphuric acid, an excess of 20 % silver nitrate solution was added, allowed to stand several hours, and filtered. A saturated solution of baryta was added to the filtrate, and the sample brought to a pH of 7–7.5, allowed to stand over night, and filtered. From the residue, silver was removed by hydrogen sulphide. To the filtrate of silver sulphide, picric acid was added. The picrate of N-methylhistidine was precipitated as the first crop, and that of imidazolepyruvic acid as the second crop. Each precipitate was recrystallized from absolute alcohol and water and its hydrochloride was also prepared. From the second crop, picric acid was removed by hydrochloric acid and benzene, and an alcoholic solution of dinitrophenylhydrazine, containing HCl was added, whereupon yellow crystals of hydrazone were precipitated.

Precipitate I: picrate: m. p. 115°–120° (moisture) 195° (decomposes)
„ „: hydrochloride: m. p. 133°–134°
N determination $C_7H_{11}O_2N_3 \cdot 2HCl + H_2O$ calculated N 16.16 %
found N 15.63 %

Precipitate II: picrate: m. p. 170°
its 2,4 dinitrophenylhydrazone m. p. 160°–161°
N determination $C_{12}H_{10}O_6N_6$ calculated N 25.14 %
found N 26.43 %

3. Enzymic experiments with DL-α-N-methylhistidine

a). 0.65 g. DL-α-N-methylhistidine was reacted for 5 days at 37° with 25 ml. rabbits or guinea-pig liver extract, which was prepared according to Takeuchi (histidase solution).

From the reaction mixture the protein was removed by tri-

cholracetic acid. No amino nitrogen was found in the filtrate. 0.792 g. DL-α-N-methylhistidine was recovered as picrates. This melted at 120° (moisture) 195° (decomp.), corresponding to 0.623 g. of free acid.

b). Demeth lase solution was prepared from 18 rabbits-kidneys, extracting with phosphate buffer solution (pH 7.1) and centrifuging. The following mixture was kept 15 hours in an incubator of 37°, boiled 20 minutes, cooled and filtered.

| | |
|---|---|
| DL-α-N-methylhistidine | 1.2448 g. |
| Demethylase solution | 170 ml. |
| Toluen | 2 ml. |

The filtrate was concentrated to 100 ml. exactly, amino nitrogen was determined with aliquot part of it and found 0.1824 mg. in total.

To a half of the reaction mixture was added histidase solution as follows:

| | |
|---|---|
| Reaction mixture | 50 ml. |
| Histidase solution | 25 ml. |
| 10% NaOH | 25 ml. (pH 9) |
| Toluen | 2 ml. |

The mixture was kept 5 days in an incubator at 37°, 30 % hydrochloric acid was added until pH 5 was reached, the mixture was warmed 20 minutes on a waterbath, cooled and filtered.

A trichloracetic acid solution was added to the filtrate, the precipitate filtered off, the filtrate was evaporated to dryness *in vacuo*, and extracted with ethanol.

The fraction soluble in ethanol was evaporated, extracted with ether to remove fatty substances, dissolved in a small amount of water, soon snow white crystals were precipitated. These melted at 255°–260°, showed Pauly's diazo reaction, but no ninhydrin reaction. The yield was 0.0146 g. and could be converted into dinitrophenylhydrazone, which melted at 158°. The fraction insoluble in ethanol was dissolved in water, added sulphuric acid and phosphotungstic acid solution as usual. The precipitate was decomposed with baryta, the baryta was removed with sulphuric acid, and picrate

was prepared. The picrate was fractionated into a small amount of urocanic acid and 0.045 g. DL-α-N-methylhistidine by recrystallization.

## Discussion

By the administration of DL-α-N-methylhistidine to a dog no urocanic acid but imidazolepyruvic acid was obtained, which has never been isolated as a product of intermediary metabolism of L-histidine. And it is very suggestive for it. The ketonic acids were also isolated from the urine of rabbits after the administration of O, N-dimethyltyrosine (To, 1939), N-methylphenylalanine (Fukuyama, 1941), N-methylvanillylalanine (Yoshida, 1941), phenylsarcosine (Oda, 1948) by coworkers of Hirohata. It was formerly accepted that the formation of ketonic acid proceeded from L-α-N-methylamino acid by the desamination of the corresponding amino acid which will be produced by the oxidation of L-α-N-methylamino acid together with formaldehyde by demethylase.

Because, first, it is confirmed by them L-abrine, viz. α-N-methyltryptophane, will be changed in rabbits body into kynurenine and kynurenic acid almost as much as tryptophane and much more than indolepyruvic acid, second: hematopoetic action of L-abrine is much more strong than indolepyruvic acid. Formation of ketonic acid from N-methylamino acid by D-amino-acid-oxidase (Keilin and Hartree 1939) and L-amino acid oxpiase (Green *et al.* 1944) has not been determined. Urocanic acid should be isolated from the urine according to Kotake *et al.*, as my dog was able to put out the acid after the subcutaneous injection of L-histidine. Failure is probably due to the weak demethylase action of the kidney of dog as demonstrated by Yosida and Fukuyama.

## Summary

1. A small amount of imidazolepyruvic acid was isolated from the urine of a dog injected subcutaneously with DL-α-N-methyl-

histidine, but no urocanic acid. It was previously confirmed that the dog was able to transform L-histidine into urocanic acid.

2. Both dogs which were investigated, were able to put out urocanic acid after the subcutaneous injection of L-histidine. 3 rabbits, however, did not do so under the same conditions.

3. Liver extract of rabbits and guinea pigs can desaminate L-histidine into urocanic acid, but not DL-α-N-methylhistidine.

4. DL-α-N-methylhistidine was reacted, at first with extract of rabbits kidney (demethylase), and subsequently that of rabbits liver (histidase), a small amount of urocanic and, imidazolepyruvic acids together with unchanged DL-α-N-methylhistidine was isolated.

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# STUDIES ON THE REACTION BETWEEN CATALASE MOLECULE AND VARIOUS INHIBITORY SUBSTANCES, I.*

By Y. OGURA, Y. TONOMURA†, S. HINO and H. TAMIYA**

(Received for publication on Feb. 23, 1950)

Catalase is an enzyme for which perhaps the largest number of inhibitory substances have thus far been known in literature. Among those substances the most studied are cyanide, hydroxylamine, azide, fluoride etc., which have been shown to change, simultaneously with their inhibitory action, the characteristic absorption spectrum of the enzyme. For this reason, these inhibitors are assumed to combine with the hemin nucleus of the catalase molecule, depriving the latter of its normal catalytic activity. However, the attempts to relate quantitatively the degree of inhibition of catalytic activity and the degree of change of absorption spectrum caused by the substances in question have so far met with but partial success or, rather, with unintelligible results (*1*).

Little attention has, on the other hand, been paid to the fact that a certain group of substances inhibit catalase activity without bringing about any modification of its spectrum. It seems reasonable to assume that these substances block a certain point in catalase molecule other than the hemin nucleus, the site of attack being most probably a certain structure of the protein moiety of the enzyme molecule.

The work that is reported below was undertaken with a view to obtaining more quantitative and systematic information as to the

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* The first report on this work was made at the Symposium on Enzyme Chemistry held by the Chemical Society of Japan in Tokyo, in September, 1946.

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reaction between catalase molecule and various inhibitory substances, using a combination of kinetic and spectrophotometric methods. Some pertinent facts which emerged from these studies may contribute to the elucidation of action mechanism and the chemical nature of catalase molecule.

## Experimental Methods.

Catalase was prepared from fresh equine liver according to the method of Kitagawa and Shirakawa (*2*), the *Cat. f.*-value (*3*) being about 20,000. Though not absolutely pure, this preparation was ascertained to contain no chromoproteids other than catalase.

(1) Kinetic method. A measured amount of hydrogen peroxide (*ca.* 0.01 mole/lit. in final experimental solution) was added to the buffered catalase solution* and, after a certain lapse of time, a definite volume of the solution was pipetted out and quickly poured into a diluted solution of sulfuric acid. The concentration of remaining hydrogen peroxide $[H_2O_2]$ was titrated with 0.01-N potassium permanganate solution. In so far as the temperature is sufficiently low and the concentration of $H_2O_2$ applied is not too high, a linear relationship is found between log $[H_2O_2]$ and the reaction time $t$, as is illustrated by line K in Fig. 1, the tangent of the line being proportional to the concentration of catalase $\varepsilon$. Form kinetic point of view, the course of decomposition of hydrogen peroxide by catalase may be represented by:

$$-\frac{d[H_2O_2]}{dt} = k\,[H_2O_2]\,\varepsilon \qquad (1)$$

therefore,

$$-\frac{d\ln[H_2O_2]}{dt} = k\,\varepsilon \qquad (2)$$

---

* In the present series of experiments 0.01 mole/lit. phosphate (pH 7.0) was used as the buffer.

where $k$ is the velocity constant of overall reaction. The tangent of the $\log[H_2O_2]$-$t$-line (K in Fig. 1) is proportional to $k\,\varepsilon$. In the presence of an inhibitor this tangent is decreased.* By denoting the tangent of $\log[H_2O_2]$-$t$-curve in the presence and absence of inhibitor with $V_G$ and $V$, respectively, we define the "degree of inhibition" ($H$) by the following equation:

$$H = 1 - \frac{V_G}{V} \qquad (3)$$

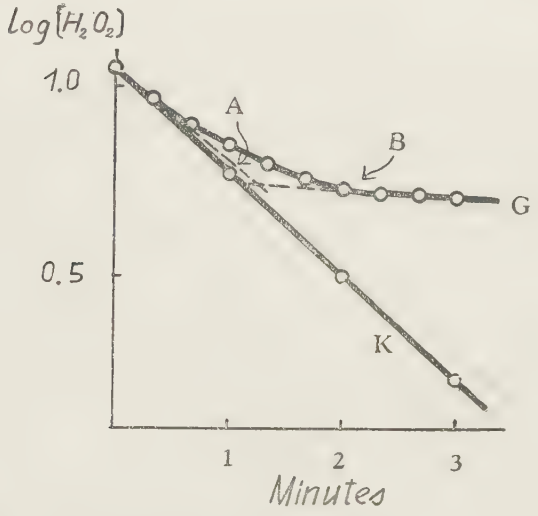


Fig. 1. Change of log $[H_2O_2]$ with time.
K: Control
G: In the presence of $10^{-7.0}$ mole/lit. $NaN_3$.

In general, $H$ varies with time, which means that, in the presence of an inhibitor, $\log[H_2O_2]$-$t$-curve does not usually represent a

* To investigate the action of inhibitors, we added to the catalase solution different concentrations of the substance to be tested and, before $H_2O_2$ was added, the mixture was allowed to stand for 15 min. at the experimental temperature so as to ensure the establishment of equilibrium between molecules of catalase and the inhibitor.

straight line but curves convexly towards the $t$-axis as represented by line G in Fig. 1; this curve can be regarded as being composed of two parts, initial and final, each representing a straight line as illustrated in Fig. 1. The states of inhibition corresponding to these two straight parts (A and B) may be called in the following the "initial" and the "final" state, respectively. It is worth noting that in each of these states the following simple relation is always established between the values $H$ and the concentration of inhibitor $G$.

$$H = \frac{G}{\phi + G} \tag{4}$$

Where $G$ is the total concentration (in mole/lit.) of inhibitor and $\phi$ (corresponding to $G$ causing 50% inhibition) is a constant characteristic to each inhibitor for its "initial" or "final" state under a definite experimental condition. If we represent graphically the relationship between $H$ and $G$ by plotting the latter on the abscissae

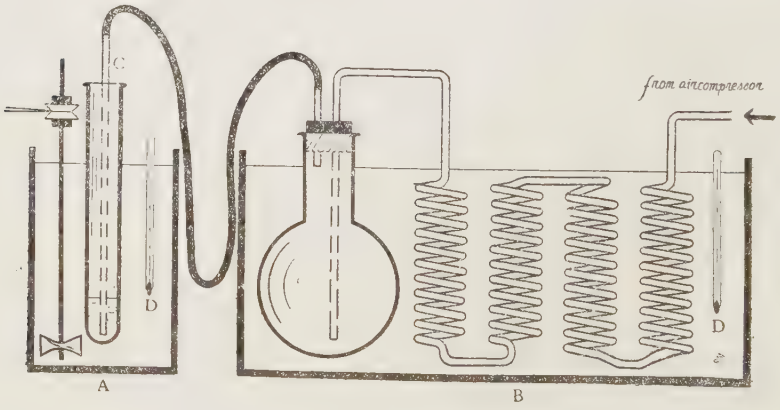


Fig. 2. Arrangement for measuring the catalase reaction at very short reaction periods.

A and B: thermostat, C: reaction tube, D: thermometer.

in logarithmic scale, we obtain a sigmoid curve in the form of a typical dissociation curve.* (In the following we denote the value $-\log G$ by "pG" and $-\log \phi$ by "p$\phi$" in analogy to the symbols "pH", "pK" etc.) These curves allow us to evaluate with satisfactory accuracy the value $\phi$ in each case.

It should be added that the duration of "initial" state or the time of transition from the "initial" to the "final" state of inhibition varies according to the inhibitor used. The method we adopted in following the initial state of most rapidly changing inhibition (*e. g.* in the case of azide) was as follows (Fig. 2). The catalase solution (9 ml. in volume) was placed in a large test tube in which a glass tube was introduced; through this tube the air which had previously been cooled to experimental temperature was passed through by means of an air-compressor. While stirring the catalase solution violently by airing, a small quantity (1 ml.) of hydrogen peroxide solution was added, and after a definite interval (*ca.* 3 seconds, which was exactly measured by a stop watch), the reaction was discontinued by pouring sulfuric acid solution into the mixture. The concentration of the peroxide remaining was titrated as usual with permanganate.

(2) Spectrophotometric method. Effect of various inhibitory substances upon the spectrum of catalase was investigated in detail using Adam Hilger's Nutting-spectrophotometer, H. 181. According to our investigations, catalase solution shows absorption maxima at

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*In the systematic study of inhibitory phenomena of various biological reactions, Tamiya *et al.* found that, quite generally, the following relationship holds between $H$ and $G$, the value $n$ being varied according to the inhibitor and the biological reaction: $H = \dfrac{G^n}{\phi^n + G^n}$. According to the value of $n$, we call the inhibitory phenomena "the inhibition of $n$-th order", and the sigmoid curves obtained as above, "the sigmoid curves of $n$-th order". The curves become considerably steeper as $n$ increases. All curves to be discussed in this paper show the inhibition of "first order" in so far as their action upon catalase is concerned.

508, 538 and 627 mμ. Change of the spectrum caused by substances capable of combining reversibly with the hemin group of catalase is generally a function of the concentration of the substance added. When the substance is added in sufficiently high concentration, a state is finally attained in which the spectrum will no more be changed by further increase of the substance. The spectrum observed at this state is to be taken as that of the catalase-inhibitor compound. When the concentration of the added substance is not high enough, the spectrum lies somewhere between those of free catalase and of the catalase-inhibitor compound. In Fig. 3 is given an example of changes in catalase spectrum caused by "saturating" ($10^{-2.0}$ mole/lit.) and "non-saturating" ($10^{-5.0}$ mole/lit.) concentrations of KCN (the concentration of catalase having been kept constant). Choosing appropriate wave length where the absorption coefficients of free and of catalase-inhibitor complex differ most widely — this "maximum" difference being denoted by $b$ —, the intermediate position, corresponding to $a$ in the figure, of the spectrum shown by non-saturating concentration of the inhibitor was determined. The ratio $a/b$, which may be denoted by $\alpha$ in the following, is a function of the concentration of the inhibitor, and investigation was made as to how this value changes with the variation of $G$.

Since both the inhibition of catalase activity and the change of spectrum may be ascribed to the combination of inhibitor molecule with the catalase, it would be of interest to compare the functional relationsphip between $H$ and $G$ with that between $\alpha$ and $G$.

For the purpose of this comparison, kinetic and spectrophotometric experiments were performed under as similar a condition as possible; namely, both at 7° and at pH 7.0 using the same concentration of buffer substance (0.01 mole/lit. phosphate). The only difference in the two cases, which was unavoidable for technical reasons, was that in test solutions applied to spectrophotometric observations the concentration of catalase was remarkably higher (*ca.* $10^{-5.0}$ mole/lit.)

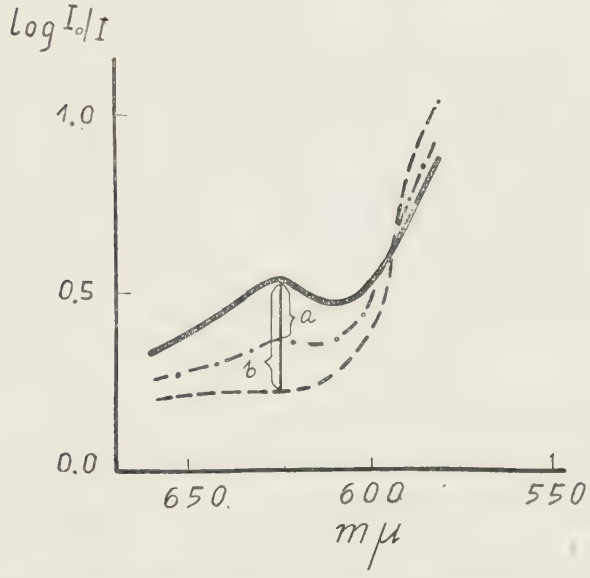


Fig. 3. Effect of addition of cyanide upon the spectrum of catalase. Concentration of catalase = *ca.* $8 \times 10^{-6.0}$ mole/lit. d = 4.0cm.
————: pure catalase, - - - -: with $10^{-2.0}$ mole/lit. KCN,
–·–·–: with $10^{-5.0}$ mole/lit. KCN.

than that in test solutions used for kinetic method (*ca.* $10^{-9.0}$ mole/lit.).

It seems relevant here to make theoretical considerations for interpreting the functional relationships to be observed between $\alpha$ and $G$, as well as that between $H$ and $G$. Let us designate the free catalase molecule with E, the molecule of the inhibitor with G, and the catalase-inhibitor complex with EG. It is reasonable to assume that E and G will react in the following manner:

$$E + G \rightleftharpoons EG$$

Designating the total concentration of catalase with $\varepsilon$, *viz.*

$$[E] + [EG] = \varepsilon \qquad (5)$$

and the dissociation constant of EG with $K_G$, *viz.*

$$K_G = \frac{[E][G]}{[EG]} \qquad (6)$$

we have

$$\frac{[EG]}{\varepsilon}=\frac{[G]}{K_G+[G]} \qquad [E]=\frac{K_G\ \varepsilon}{K_G+[G]} \tag{7}$$

On the other hand, the total concentration of inhibitor applied is

$$G=[G]+[EG] \tag{8}$$

It may be clear that the ratio $a/b$ determined in spectrophotometric experiments is the ratio $[EG]/\varepsilon$, *viz.*

$$\alpha=\frac{[EG]}{\varepsilon} \tag{9}$$

Considering Eqs. (7), (8) and (9), we have

$$\frac{(I-\alpha)\ (G-\alpha\ \varepsilon)}{\alpha}=K_G \tag{10}$$

If the concentration of catalase is very low compared with that of the inhibitor, so that $\alpha\varepsilon$ may be considered negligible against $G$, Eq. (10) will be transformed into

$$\alpha=\frac{G}{K_G+G} \tag{11}$$

As will be shown later, the $\alpha$-pG-curve obtained with cyanide and azide corresponds satisfactorily to Eq. (10) and deviates widely from Eq. (11), indicating that thereby the value $\alpha\varepsilon$ has been of appreciable magnitude compared with $G$. Itshould be noted that by analysis of experimental $\alpha$-pG-curve on the basis of Eq. (10), we can determine the values $\varepsilon$ and $K_G$.

Of great interest is the comparison of $K_G$ thus found with the volues of $\phi$ which were determined by kinetic experiments. It may be quite natural to assume that, at least under certain conditions, the velocity of $H_2O_2$ decomposition in the presence of an inhibitor may be determined by the concentration of free catalase $[E]$ as it is given by Eq. (7). Under such conditions the degree of inhibition, as it may be derived from the equation given already, will be

$$H=\frac{[G]}{K_G+[G]}$$

considering that in kinetic experiments the concentration of catalase applied was very low, we may assume that thereby $[EG]$ would have been negligibly small compared with $[G]$. Equating, therefore,

[G] to $G$, we have

$$H = \frac{G}{K_G + G} \qquad (12)$$

a relation which is formally the same as Eq. (4). Under the said condition the value $\phi$ must be equal to $K_G$; indeed, as will be shown later, with all inhibitors that modify the spectrum of catalase it was found that the value $\phi$ at the inital state was equal to $K_G$ which was determined by the analysis of $\alpha$-pG-curve obtained by spectrophotometric method. The interesting question as to how to interpret the $\phi$-value found at the final state will be discussed later.

## Results.

### (1) *Inhibitors modifing the spectrum of catalase.*

Following substances modify more or less the spectrum of catalase in the visible region: cyanide, chloride, fluoride, sulfide, monomethyl hydrogen peroxide, azide and hydroxylamine. Absorption maxima of various catalase-inhibitor complexes are given in Table I, and absorption spectra of some representative compounds are illustrated in Fig. 4. In their inhibitory action each of these substances showed more or less a specific phenomenon, typical features of which were found in cyanide and azide, which are described in the following.

Table I

*Absorption maxima of various catalase compounds*

| Compounds | Absorption-maxima (mμ) |
|---|---|
| Catalase | 508, 538, 625 |
| Cyanide-catalase | 544, 575 |
| Fluoride-catalase | 505, 540, 606, 620 |
| Azide-catalase | 540, 623 |
| Hydroxylamine-catalase | 507, 540, 625, 640 |

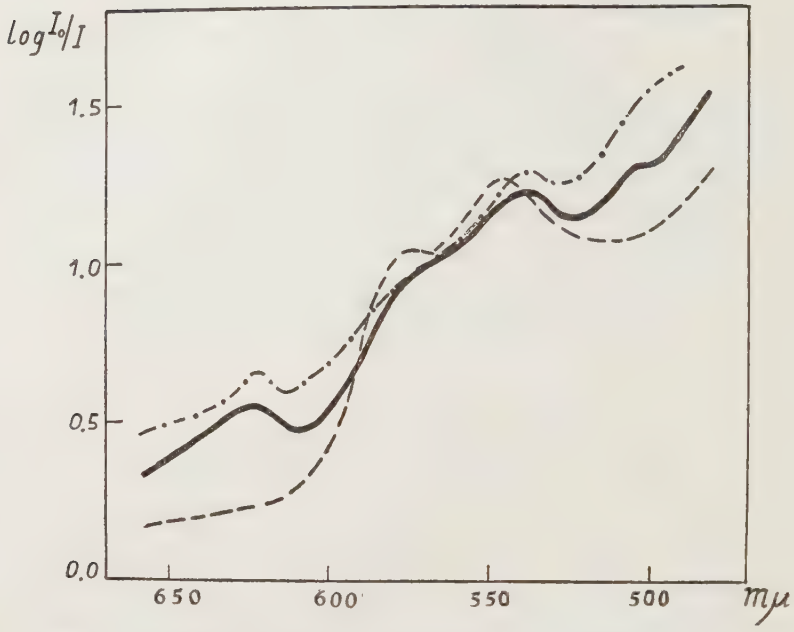


Fig. 4. Spectra of cyanide- and azide-catalase compared with that of free catalase.
$\varepsilon = ca.\ 8\times10^{-6.0}$ mole/lit. d = 4.0 cm.
——: catalase, – – – –: cyanide-catalase, –·–·–·–: azide-catalase.

(a) Cyanide. As was mentioned already, the $\log[H_2O_2]$-$t$-curve, in the presence of an inhibitor, generally bends convexly towards the $t$-axis. The only exception to this general rule was found in the case of cyanide, in which the value $\phi$ remained constant throughout the course of reaction. The $\phi$-value found was $10^{-6.2}$ mole/lit. at 0°, $10^{-6.15}$ mole/lit. at 1°, and $10^{-5.8}$ mole/lit. at 5°. (See protocol 1).

By spectrophotometric experiments the functional relationship between $\alpha$ and $G$ was investigated and it was found to follow Eq. (10) instead of Eq. (11) (See Fig. 6, Protocol 2). Analysis of the curve on the basis of Eq. (10) showed that $\varepsilon$ was $1.68\times10^{-5.0}$ mole/lit. and $K_G$ was $10^{-5.8}$ mole/lit. at 5°. This value of $K_G$

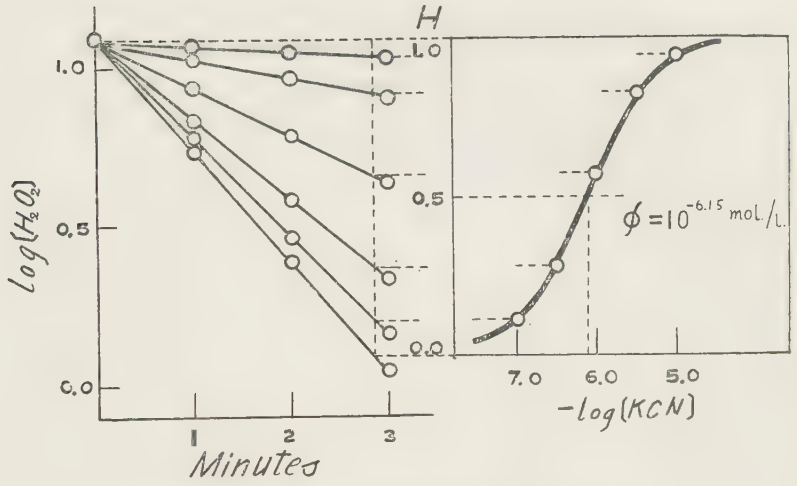


Fig. 5. Determination of the $\phi$-value of KCN at pH 7.0, 1°.
Left: $\log [H_2O_2]$-t relation, Right: $H$-pG-curve.

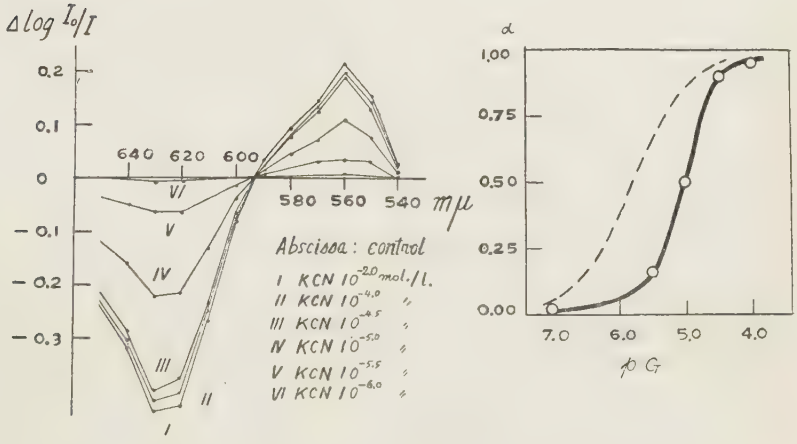


Fig. 6. Determination of the $K_G$ value of KCN at pH 7.0, 5°.

coincides well with the $\phi$-value estimated by the kinetic method.

(b) Azide. This substance, as all the others to be described later, shows two distinct phases of inhibition, "initial" and "final", as is illustrated in Fig. 1. That the inhibition-pG-curves in both of

these phases represent a sigmoid of the first order is shown in Fig. 7, and Fig. 9. The conspicuous difference in the lateral position of the two curves corresponds to the difference between the initial and final $\phi$-values which, together with the values found for other inhibitors, are given in Table II.

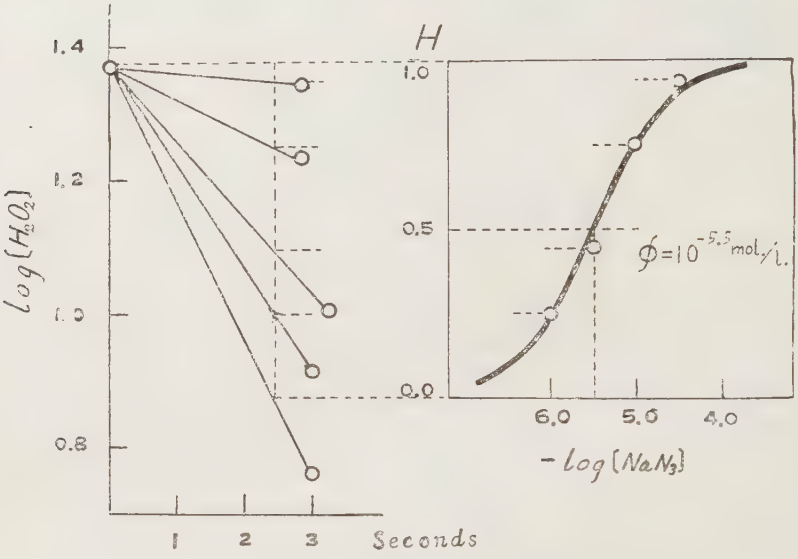


Fig. 7. Initial value of $\phi$ of $NaN_3$ at pH 7.0, 7°.

Spectrophotometric investigation analogous to that performed with cyanide showed that in this case also the relation between $\alpha$ and $G$ assumed the form of Eq. (10). By analysis of the curves obtained, $\varepsilon$ was found to be $2.5\times10^{-6.0}$ mole/lit. and $K_G=10^{5.7}$ mole/lit. at 7°. The latter value agrees exactly with $\phi$ of the initial state observed in kinetic experiments.

Of the observations we have made with azide, the following might perhaps be most significant in connection with the question of how to interpret the remarkable bending of the $\log[H_2O_2]$-$t$-curves giving rise to the final state of inhibition. The $\log[H_2O_2]$-$t$-curve bending in the manner illustrated by curve G in Fig. 1 is always

TABLE II

*$pK_G$ and $p\phi$ of inhibitors modifying the spectrum of catalase at pH 7.0.*

| Inhibitor | $pK_G$ (5°) | $p\phi$ | |
|---|---|---|---|
| | | Initial (7°) | final (0°) |
| KCN | 5.8 | 5.8 (5° C.) | |
| NaF | 1.8 | 1.8 | 2.3 |
| $Na_2S$ | — | 4.0 | 5.6 |
| $CH_3OOH$ | — | 5.8 | — |
| $NaN_3$ | 5.7 | 5.7 | 7.7 |
| $NH_2OH$ | 5.0 | 4.9 | 6.9 |

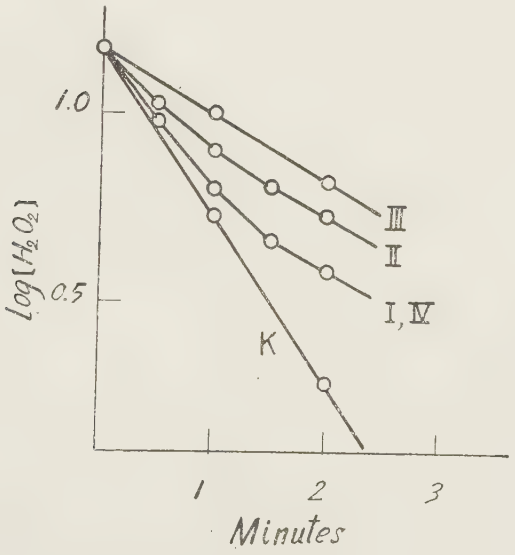


Fig. 8. Influence of pretreatment with $H_2O_2$ on the initial state of $NaN_3$ ($10^{-7.5}$ mole/lit.) inhibition.
K: Control, I: without pretreatment, II: experiment conducted 1 minute after pretreatment, III & IV : experiments conducted 5 and 25 minutes, respectively, after pretreatment.

obtained when catalase, previously set in equilibrium with the inhibitor, is allowed to act upon $H_2O_2$ (given in a concentration of ca. 0.01 mole/lit. in the final mixture) and the course of $H_2O_2$ decomposition was followed from the very moment of the addition of $H_2O_2$. When, however, catalase is pretreated with a very small quantity of $H_2O_2$ (ca. 0.0005 mole/lit. in the final experimental solution) before the usual quantity of $H_2O_2$, whose rate of decomposition is to be followed, is added, interesting phenomena are observed as shown in Fig. 8. If the interval between the pretreatment, *i. e.* the addition of a very small quantity of $H_2O_2$, and the addition of $H_2O_2$ to be measured is about 5 minutes, the initial state completely disappears and the $\log[H_2O_2]$-$t$-curve takes a straight course showing a tangent that is exactly the same as that of the "final" state; in other words, the curve shows the "final" state from the beginning. If the interval in question is shorter or longer than 5 minutes (say 1 minute or 10 minutes) the initial phase does appear more or less recognizably, the distinctness of which depending upon how much the interval deviates from 5 minutes. When the interval is made sufficiently long, *e. g.* 25 minutes, the effect of pretreatment completely disappears, so that the $\log[H_2O_2]$-$t$-curve assumes the same form as in

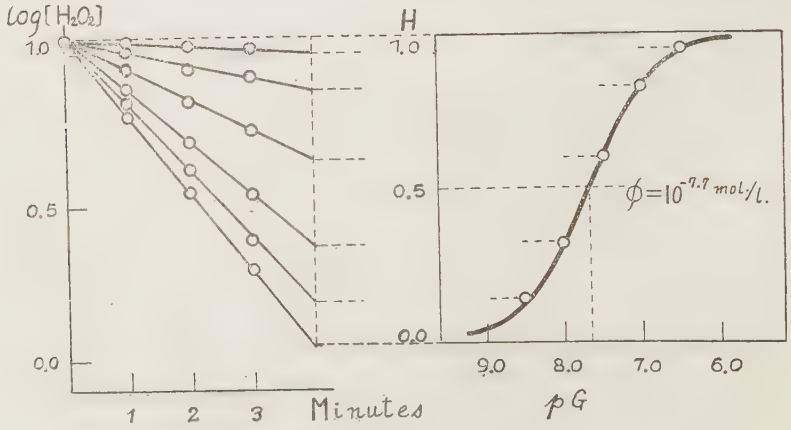


Fig. 9. Final value of $\phi$ of $NaN_3$ at pH 7.0, 0°.

the case without pretreatment.

From these observations it may be concluded that; (1) the final state of inhibition is the state that is realizable only in the presence of $H_2O_2$, the concentration of which may be as low as 0.0005 mole/lit.; (2) for the establishment of the final state a certain reaction time is necessary which is about 5 minutes in the case of azide; and (3) the establishment of final state is a reversible process, the return to the original state after 25 minutes being explicable by the disappearance of $H_2O_2$ owing to its decomposition by the activity of catalase. Later it will be shown that these findings provide an important clue to an understanding not only of inhibitory phenomena but also of some aspects in the mechanism of normal process of $H_2O_2$ decomposition by catalase.

(c) Fluoride, Sulfide and Hydroxylamine. Similar experiments, kinetic and spectrophotometric, as described above were carried out with fluoride, sulfide and hydroxylamine, which all change the absorption spectrum of catalase by reacting with it. These inhibitors also share the property of showing initial and final state of inhibition which is singularly lacking in cyanide. The value of $pK_G$ and $p\phi$ found are summarized in Table II, from which it may be seen that the initial $\phi$-values are always equal to $K_G$ and are more or less larger than the corresponding values in the final state.

(2) *Inhibitors not modifying the spectrum of catalase.*

We found that the following substances inhibit the activity of catalase without modifying its absorption spectrum: phenol, resorcinol, hydroquinone, cresols, chlorophenols, nitrophenoles and hydrogen ion. In the following points, however, all these inhibitors show the same phenomena as the spectrum-modifying inhibitors which have been discussed already; (1) they give the $\log[H_2O_2]$-$t$-curve bending convexly towards the $t$-axis, showing initial and final states of inhibition; (2) in both of these states, the inhibition-pG-curve represents a typical sigmoid of the first order; and (3) by pretreatment

with a very small quantity of $H_2O_2$ the initial states disappears, the $\log[H_2O_2]$-$t$-curve taking "final" course from the beginning.

The values of $p\phi$ obtained with some typical substances belong-

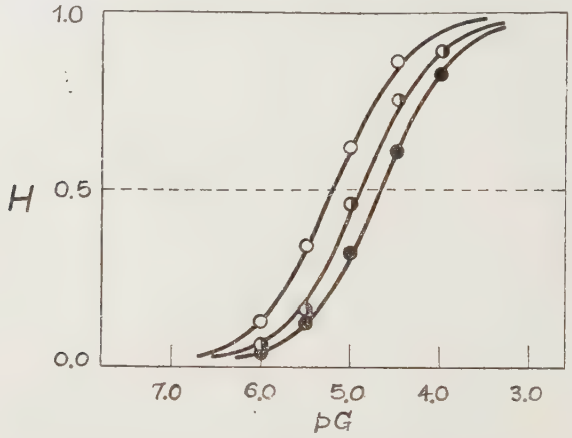


Fig. 10. $H$-pG-curve for some phenolic substances (final state of inhibition at pH 7.0, 0°.)
—○— $o$-chlorophenol —◑— hydroquinone —●— $p$-cresol.

TABLE III

*$\phi$-values of inhibitors not modifying the spectrum of catalase.*

| Inhibitor | $p\phi$ | |
|---|---|---|
| | Initial (7°) | Final (0°) |
| HO—C$_6$H$_4$—$CH_3$ | 2.1 | 4.6 |
| HO—C$_6$H$_3$(Cl)— | 2.1 | 5.2 |
| HO—C$_6$H$_4$—OH | — | 4.9 |
| $H^+$ | 4.5 | 5.8 |

ing to this group are given in Table III. The *H*-pG-curves shown by these substances (in their final states of inhibition) are illustrated in Fig. 10. The fact that the substances of this group do not modify the spectrum of catalase indicates that they exert no influence upon the energy levels of protohemins. The observation of ultraviolet and infrared regions of spectrum* was carried out using *p*-cresol but even in its highest possible concentration ($10^{-1.8}$ mole/lit.) we could not confirm any sign of modification.** It may be certain that the site of attachment of these substances is some group in the protein moiety of the catalase molecule. The *H*-pG-curve shown by hydrogen ion is of special theoretical interest; it will be given and discussed in detail in our later report.

## Discussion.

From the facts described in detail in connection with the action of azide, it may be inferred that the "final" state is connected with the reversible combination of catalase molecule with $H_2O_2$. This reaction may be pictured by

$$E + S \rightleftarrows \overset{S}{E}$$

where S is $H_2O_2$ and $\overset{S}{E}$ the catalase-$H_2O_2$-complex in question. As it has been shown with various inhibitors, the $\phi$-value observed in the "initial" state may be regarded as the dissociation constant of the combination between E and G:

$$E + G \overset{K_G}{\rightleftarrows} EG \qquad (I)$$

The most plausible explanation for the $\phi$-value found in the "final" state may be that it represents the dissociation constant of the combination between $\overset{S}{E}$ and G, *viz.*

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*For measurement of absorption coefficients in infrared region we are indebted to Dr. Shimanouchi, Chemical Institute, University of Tokyo.

**The concentration of this substance cannot be made higher than $10^{-1.5}$ mole/lit., because irreversible destruction of catalase occurs and the solution becomes turbid.

$$\overset{S}{E} + G \overset{K'_G}{\rightleftarrows} \overset{S}{E}G \qquad \text{(II)}$$

The dissociation constant in question may be denoted by $K'_G$.

Based on this idea we may picture the mechanism of the catalase reaction in the following manner:

$$E + S \underset{k'_1}{\overset{k_1}{\rightleftarrows}} \overset{S}{E} \qquad \text{(III)}$$

$$\overset{S}{E} + S \underset{k'_2}{\overset{k_2}{\rightleftarrows}} S\overset{S}{E} \qquad \text{(IV)}$$

$$S\overset{S}{E} \xrightarrow{k_3} E + P \qquad \text{(V)}$$

where $S\overset{S}{E}$ is a compound in which two molecules of $H_2O_2$ are combined with one molecule of catalase, and P the reaction products, *viz.* $O_2$ and $H_2O$. The values $k$, $k'$, etc. are velocity constants of the reactions towards the direction indicated by the arrow. Considering a number of experimantal facts thus far known and others which will be referred to in our later reports, it is assumed that; the equilibrium of the reaction (III) is shifted extremely to the right, and can be attained very rapidly. From this assumption can be deduced that within a certain range of $H_2O_2$ concentration, *i.e.* when $\frac{k'_2+k_3}{k_2} \gg [S] \gg \frac{k'_1}{k_1}$, practically all catalase molecules will take the form of $\overset{S}{E}$ and the velocity, $v$, of overall reaction may be given by

$$v = \frac{2\,d\,[P]}{dt} = \frac{2\,k_2\,k_3}{k'_2+k_3}\,\varepsilon\,[H_2O_2]$$

This equation coincides with the experimental formula (1), indicating that the overall rate constant $k$ is $\frac{2\,k_2\,k_3}{k'_2+k_3}$.

In the presence of an inhibitor G, the reactions (I) and (II) will occur besides the above-mentioned three. It is assumed that in most of the inhibitors studied $K_G$ is larger than $K'_G$, and the equilibrium of these reactions can not be attained instantaneously.

In the absence of $H_2O_2$, reaction (I) represents the only reaction

to occur, and at the equilibrium the relation:

$$\frac{[EG]}{\varepsilon}=\frac{[G]}{K_G+[G]} \qquad \frac{[E]}{\varepsilon}=\frac{K_G}{K_G+[G]} \tag{13}$$

is fulfilled. Obviously, it is the equilibrium of this reaction that was observed in spectroscopic experiments.

Now, let us consider what events will occur in kinetic experiments. In all our kinetic experiments, the catalase and inhibitor were at first set in equilibrium for sufficient period of time before $H_2O_2$ was added and its rate of decomposition was followed. On addition of $H_2O_2$, the reaction (III) followed by (IV) and (V) will suddenly occur besides the preexisting system (I). Owing to the formation of $\overset{S}{E}$ by reaction (III) there will appear the possibility of the reaction (II). As was mentioned already, however, both reactions (I) and (II) occur rather slowly and the shift of the equilibrium of reaction (I) can take place only sluggishly. Within the time in which the occurrence of reaction (II) is negligible, the velocity ($v_G$) of $H_2O_2$ decomposition will be limited by the concentration of free catalase that is given by Eq. (13), *viz.*

$$v_G=k\,[E]\,[H_2O_2]=\frac{k\,K_G}{K_G+[G]}\,\varepsilon\,[H_2O_2]$$

Considering that in kinetic experiments [G] may be equated to $G$, the apparent velocity constant $V_G$, and the degree of inhibition ($H$) observed under the said condition are given by

$$V_G=\frac{K_G}{K_G+G}\,V$$

$$H=1-\frac{V_G}{V}=\frac{G}{K_G+G} \tag{14}$$

These equations correspond to the fact that in initial state the inhibition-pG-curve represents a typical sigmoid of the first order, giving the $\phi$-value which is equal to $K_G$ determined by spectrophotometric method.

With the lapse of time the reaction (II) gradually takes place and the preexisting equilibrium (I) is disturbed owing to the disappearance of the free form E of catalase as a result of reaction

(III) in which the equilibrium is shifted extremely to the right. When eventually a final state is attained, the velocity of $H_2O_2$ decomposition will become

$$v_G = \frac{k_2\, k_3\, K'_G\, \varepsilon\, [H_2O_2]}{(k'_2+k_3)\,(K'_G+G)}$$

so that the degree of inhibition ($H'$) at this state will be given by

$$H' = \frac{G}{K'_G+G} \qquad (15)$$

This equation fits in well with the experimental fact that in the "final" state also, the inhibition-pG-curve represents a typical sigmoid of the first order with the $\phi$-value different from that of the "initial" state. The fact that $\phi$ in the final state is smaller than that in the initial state means that $\overset{S}{E}$ has a stronger affinity to the inhibitor molecule than the free catalase E.

As was mentioned already, all these considerations are referred to the condition: $\frac{k'_2+k_3}{k_2} \gg [S] \gg \frac{k'_1}{k_1}$, and in so far as this condition is fulfilled the $\phi$-values must be independent of the concentration of peroxide used. That this is actually the case was confirmed with various inhibitors (cyanide, azide and *o*-chlorophenol), the $\phi$-values of which remained constant when peroxide concentration was varied from $10^{-2.7}$ to $10^{-1.3}$ mole/lit.

In view of the fact that the $\phi$-values in the initial state could indubitably be correlated to the $K_G$-value measured spectrophotometrically, similar experiments to check the final $\phi$-value by spectrophotometric observation appeared to be of interest and worthy of undertaking. Unfortunately, such experiments were not feasible owing to the fact that, since we had to use high concentration of catalase in spectrophotometric experiments, hydrogen peroxide added was almost instantaneously decomposed even when the inhibitors were added in considerably high concentrations. The spectroscopic observation of the combination between E and G may only be possible with spectrophotometer of extraordinary sensitivity and with extraordinary rapidity of action, which seems to be beyond

the technical possibility at present.*

## Summary.

1) The reaction between catalase molecule and various inhibitory substances was studied quantitatively by using both kinetic and spectrophotometric methods.

2) The characteristic spectrum of catalase is changed by its combination with cyanide, chloride, fluoride, sulfide, azide and hydroxylamine, while no change could be observed by that with phenol, resorcinol, hydroquinone, cresols, chlorophenols, nitrophenols and hydrogen ion, though these strongly inhibit catalase activity. Substances of the latter group may be regarded as exerting their inhibitory action by combining with certain structual element in protein moiety of the catalase molecule.

3) In the absence of inhibitors, the $\log[H_2O_2]$-time-curve observed in the catalatic $H_2O_2$-decomposition represents, as is well known, a straight line at lower temperatures and within certain limits of $H_2O_2$ concentration applied. In the presence of inhibitors, however, the $\log[H_2O_2]$-time-curve usually bends more or less convexly towards the time-axis. The exception was found only in cyanide in which the $\log[H_2O_2]$-time-curve represented a straight line as in the control experiment. The bending curves were found to be composed of two straight lines with different tangents, and for the corresponding two states of inhibition, referred to as the

* After we finished these series of experiments, we learned that similar experiments on the inhibition phenomena of catalase had been reported by Agner and Theorell in 1946. Their experiments were carried out at 20°, and the reaction time lasted 8-10 minutes. Undoubtedly, they had observed highly complicated phenomena in which the initial and final states in our sense must have been entangled irregularly. As a result the inhibition-pG-curves obtained by their experiments markedly deviate from the typical sigmoid of the first order. The explanation of this fact attempted by the said authors seems to us to be too much of an "*ad hoc*" to be acceptable from kinetic point of view.

"initial" and "final" states of inhibition, the following relationship between the degree of inhibition ($H$) and concentration of inhibitor applied ($G$) was found to hold with sufficient accuracy:

$$H = \frac{G}{\phi + G}$$

The value $\phi$ which represents the inhibitor concentration causing 50% inhibition was found to be always larger in the "initial" state than in the "final" state.

4) The dissociation constants of each catalase-inhibitor-complex, which could be determined by spectrophotometric method were always found to be identical with the "initial" $\phi$-values obtained by kinetic method.

5) It was found that on addition of a small quantity of hydrogen peroxide before experiment the initial state disappears, the $\log[H_2O_2]$-time-curve showing the "final" state from the beginning.

6) All these facts could satisfactorily be accounted for by the following assumptions: catalase molecule (E) first reacts with a molecule of hydrogen peroxide (S) in the manner

$$E + S \rightleftharpoons \overset{S}{E} \qquad (I)$$

where $\overset{S}{E}$ represents the primary complex built between E and S. $\overset{S}{E}$ then will react with another molecule of S according to the equation

$$\overset{S}{E} + S \rightleftharpoons S\overset{S}{E} \qquad (II)$$

and the resulting complex $S\overset{S}{E}$ will finally decompose, yielding free catalase (E) and the reaction products,

$$S\overset{S}{E} \longrightarrow E + O_2 + H_2O \qquad (III)$$

It was further assumed that; the equilibrium of reaction (I) is shifted extremely to the right, and can be attained very rapidly. According to this assumption, practically all catalase molecules will be in the state $\overset{S}{E}$ under stationary condition, and the velocity of the overall reaction $v$ will be given by

$$v = k \varepsilon [H_2O_2]$$

which is in accordance with the fact that in the absence of inhibitors the $\log[H_2O_2]$-time-curve represents a straight line.

In the presence of an inhibitor the following two reaction will occur besides the above-mentioned three:

$$E + G \rightleftharpoons EG \qquad \text{(IV)}$$

$$\overset{S}{E} + G \rightleftharpoons \overset{S}{E}G \qquad \text{(V)}$$

Assuming that both these reactions can take place rather slowly, it was deduced that the $\phi$-values found in the "initial" and "final" states are the dissociation constants of EG and $\overset{S}{E}G$, respectively, the former having larger dissociation constant than the latter. Among all the inhibitors studied, cyanide occupies a special position, in which reactions (IV) and (V) seem to occur very rapidly with the dissociation constants not differing from one another.

This investigation forms a part of the study of the Research Commitee of Heavy Metal Catalysis of the National Research Council and was supported by a grant donated to one of the authors (Tamiya) by the Ministry of Education.

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PROTOCOL 1

*Inhibition of catalase activity by cyanide*

Composition of the experimental solution was as follows: catalase solution 2 ml.; 1/15 mole/lit, phosphate buffer (pH 7.0) 2 ml.; potassium cyanide solution or redistilled water 2 ml.; redistilled water 12 ml. Temperature: 1°. After thermal equilibrium was attained, 2 ml. of hydrogen peroxide solution was added, and after 1, 2 and 3 minutes, 5 ml each of the solution were withdrawn and poured into a 25% sulfuric acid solution; titration with N/100 potassium permanganate gave the following results. $H_{obs.}$ is the degree of inhibition determined from the $\log[H_2O_2]$-$t$-curves and $H_{cal.}$ is the corresponding value calculated by means of Eq. (4), assuming the $\phi$-value of $10^{-6.15}$ mole/lit.

| $-\log$ [KCN] | N/100 permanganate consumed (ml.) | | | $H_{obs.}$ (%) | $H_{cal.}$ (%) |
|---|---|---|---|---|---|
| | 1 min. | 2 min. | 3 min. | | |
| $\infty$ | 5.45 | 2.50 | 1.10 | 0 | 0 |
| 7.0 | 6.00 | 2.90 | 1.45 | 12 | 12 |
| 6.5 | 6.75 | 3.80 | 2.20 | 29 | 31 |
| 6.0 | 8.60 | 6.10 | 4.35 | 57 | 57 |
| 5.5 | 10.75 | 9.35 | 8.15 | 82 | 81 |
| 5.0 | 11.75 | 11.25 | 10.55 | 95 | 93 |

PROTOCOL 2.

*Change of catalase spectrum caused by cyanide*

As the light source, a Mazda projector-lamp (100V-500W) was used at a suitable voltage. Light beam was filtered through Riken Ultrasin Filter No. 3. Absorption coefficients at 630 and 560 mμ were measured. Temperature: 5°. $\alpha_{obs.}$ are observed values and $\alpha_{cal.}$ are the corresponding value calculated by means of Eq. (11), assuming $K_G$ and $\varepsilon$ to be $10^{-5.8}$ and $1.68\times10^{-5.0}$ mole/lit., respectively.

| $-\log$ KCN | $\alpha_{obs.}$ | $\alpha_{cal.}$ |
|---|---|---|
| 2.0 | 1.0 | 1.0 |
| 4.0 | 0.95 | 0.97 |
| 4.5 | 0.90 | 0.91 |
| 5.0 | 0.50 | 0.50 |
| 5.5 | 0.17 | 0.13 |

PROTOCOL 3.

*Initial state of inhibition caused by azide*

Experimental solutions used; 1/15 mole/lit. phosphate buffer (pH 7.0) 1 ml.; catalase solution 1 ml. (about 40 times as high as in the experiment of protocol 1); sodium azide solution or redistilled water 1 ml.; redistilled water 6 ml. When thermal equilibrium was attained, 1 ml hydrogen peroxide solution was added quickly, stirring violently with the aid of an aircompressor. After about 3 seconds, during which time the solution was constantly mixed by air, 10 ml. of sulfric acid solution were poured into the mixture, and the concentration of remainning peroxide was titrated with N/100 permanganate, $H_{cal.}$ is the degree of inhibition by means of Eq. (4), assuming the $\phi$-value of $10^{-5.7}$ mole/lit.

| $-\log [NaN_3]$ | N/100 permanganate consumed | | | | $H_{obs.}$ (%) | $H_{cal.}$ (%) |
|---|---|---|---|---|---|---|
| | Time (sec.) | ml. | Time (sec.) | ml. | | |
| $\infty$ | 0 | 23.4 | 3.0 | 5.95 | 0 | 0 |
| 6.0 | „ | „ | 3.0 | 8.2 | 25 | 24 |
| 5.5 | „ | „ | 3.2 | 10.4 | 45 | 50 |
| 5.0 | „ | „ | 3.15 | 17.1 | 75 | 75 |
| 4.5 | „ | „ | 3.15 | 22.0 | 94 | 91 |

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# CLASSIFICATION OF CATALASE-POISONS BASED ON OBSERVATIONS OF THEIR INTERACTION WITH CATALASE.* II.

By

Y. OGURA, Y. TONOMURA†, S. HINO and H. TAMIYA.**

(Received for publication on Feb. 23, 1950)

In the previous paper (1) we reported on the inhibition phenomena of catalase reaction caused by various poisonous substances and showed that one can distinguish two different states, "initial" and "final", which manifest themselves in the gradual change of inhibitory grade with the progress of $H_2O_2$ decomposition. Observations made both with kinetic and spectrophotometric methods have led us to the conclusion that in the course of catalase reaction catalase molecule (E) transforms into a certain form ($\overset{S}{E}$) which was assumed to be a complex in which $H_2O_2$ is reversibly associated with the catalase molecule. The "initial" and "final" states of inhibition were explained as being due to the action of inhibitor substance upon E and $\overset{S}{E}$, respectively. Based on this inferrence we estimated, for each inhibitory substance, dissociation constants of the complexes built of the inhibitor and the two forms of catalase.

The present studies are concerned with the phenomena which occur when two different kinds of inhibitors are added to the catalase. As will be described, there are several kinds of interactions between inhibitors in their action upon the catalase, and by quantitative analyses of the phenomena it was revealed that there are at least four different sites in a catalase molecule on which different groups of inhibitors attach, exerting more or less profound

* This first report on this work was made at the Symposium on Enzyme Chemistry held by the Chemical Society of Japan in Tokyo, in September 1946.

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influences upon the reaction of other inhibitors.

## Theory.

Interactions between two poisons acting upon catalase can be studied by kinetic as well as spectrophotometric method, the latter being, however, applicable only to those inhibitors which modify the spectrum of the catalase. Before presenting experimental results obtained by these two methods, it seems advisable to give some theoretical considerations dealing with the phenomena in general to be expected in the simultaneous presence of two different inhibitors.

(I) Possible phenomena to be observed by kinetic method.

Let us designate the molecule of catalase by E and the two poisons to be given together by $G_1$ and $G_2$. Each of these inhibitors will react with E according to the equation:

$$E + G_1 \overset{K_{G_1}}{\rightleftharpoons} EG_1 \qquad (1)$$

$$E + G_2 \overset{K_{G_2}}{\rightleftharpoons} EG_2 \qquad (2)$$

Besides these reactions we have to assume that, if the sites of attachment of the two substances to the catalase molecule are different, one molecule of catalase might be able to combine with both of these substances at the same time. Simultaneous combination of the two inhibitors may be expressed by;

$$EG_2 + G_1 \overset{K_{G'_1}}{\rightleftharpoons} EG_1G_2 \qquad (3)$$

$$(\text{or } EG_1 + G_2 \overset{K_{G'_2}}{\rightleftharpoons} EG_1G_2)$$

The values $K_G$'s given in the equations are the dissociation constants of the respective reactions. If we denote the total concentration of catalase by $\varepsilon$, it can be shown that

$$\varepsilon = [E](1+[G_1]/K_{G_1}+[G_2]/K_{G_2}+ K_{G_1}/K_{G'_1} \cdot [G_1]/K_{G_1} \cdot [G_2]/K_{G_2}) \qquad (4)$$

The velocity of catalase reaction which will be observed in the

"initial" state is proportional to the concentration E of the catalase; denoting this velocity to be observed in the presence of $G_1$ and $G_2$ by $v_{1,2}$, we have

$v_{1,2}=k\,[E]\,[H_2O_2]=$

$$\frac{k\varepsilon\,[H_2O_2]}{1+[G_1]/K_{G1}+[G_2]K_{G2}+(K_{G1}/K_{G'1}\cdot[G_1]/K_{G1}\cdot[G_2]/K_{G2})} \tag{5}$$

where $k$ is the velocity constant of the overall reaction. In the presence of $G_1$ alone, the velocity ($v_1$) to be observed will be

$$v_1=\frac{k\varepsilon\,[H_2O_2]}{1+[G_1]/K_{G1}} \tag{6}$$

Let us consider the phenomena to be observed when the concentration of $G_2$ is varied, while that of $G_1$ is kept constant. In this case the degree of inhibition ($H_{\bar{1},2}$) referred to $G_2$ may be defined by

$$H_{\bar{1},2}=1-\frac{V_{\bar{1},2}}{V_1} \tag{7}$$

where $V$'s are apparent velocity constants, that is, $V=v/[H_2O_2]$. Taking into consideration the relations given above, we have

$$H_{\bar{1},2}=\frac{G_2}{\phi_{\bar{1},2}+G_2} \tag{8}$$

where

$$\phi_{\bar{1},2}=K_{G2}\left\{\frac{\dfrac{K_{G'1}}{K_{G1}}\,(K_{G1}+G_1)}{\dfrac{K_{G'1}}{K_{G1}}\cdot K_{G1}+G_1}\right\} \tag{9}$$

Eq. (8) shows that the $H_{\bar{1},2}$-pG-relation will give a "sigmoid curve of the first order".

Similar equations can be derived for the "final" state of inhibition, in which, as was discussed already, the inhibition concerns with the reaction between inhibitors and the intermediate $H_2O_2$-catalase complex which we had designated by $\overset{S}{E}$. In analogy to the equations already given, we may picture the following reactions:

$$\overset{S}{E}\quad+\quad G_1\quad\underset{\rightleftharpoons}{K'_{G1}}\quad\overset{S}{E}G_1 \tag{10}$$

$$\overset{S}{E} + G_2 \overset{K'_{G_2}}{\rightleftharpoons} \overset{S}{E}G_2 \qquad (11)$$

$$\overset{S}{E}G_2 + G_1 \overset{K'_{G'_1}}{\rightleftharpoons} \overset{S}{E}G_1G_2 \qquad (12)$$

$$(\text{or } \overset{S}{E}G_1 + G_2 \overset{K'_{G'_2}}{\rightleftharpoons} \overset{S}{E}G_1G_2)$$

The degree of inhibition ($H'_{\bar{I},2}$) referred to $G_2$ to be observed in the "final" state will be

$$H'_{\bar{I},2}=1-\frac{V_{\bar{I},2}}{V_1}=\frac{G_2}{\phi'_{\bar{I},2}+G_2} \qquad (13)$$

where

$$\phi'_{\bar{I},2}=K'_{G2}\left\{\frac{\frac{K'_{G'_1}}{K'_{G_1}}(K'_{G_1}+G_1)}{\frac{K'_{G'_1}}{K'_{G_1}}\cdot K'_{G_1}+G_1}\right\} \qquad (14)$$

Now, concerning the mutual actions between $G_1$ and $G_2$ we can consider the following four possibilities.

1) The two poisons combine "competitively" with the same active center of the catalase, so that the formation of complexes $EG_1G_2$ or $\overset{S}{E}G_1G_2$ is precluded. In this case

$$K_{G'_1}/K_{G_1}=\infty \quad \text{and} \quad K'_{G'_1}/K'_{G_1}=\infty$$

accordingly,

$$\phi_{\bar{I},2}=K_{G_2}(1+G_1/K_{G_1}) \text{ and } \phi'_{\bar{I},2}=K'_{G_2}(1+G_1/K'_{G_1}). \qquad (15)$$

2) The two poisons combine with different active centers of the catalase, exerting no influence at all upon each other. In this case the formation of $EG_1G_2$ and $\overset{S}{E}G_1G_2$ is possible, whereby

$$K_{G_1}=K_{G'_1} \text{ and } K'_{G_1}=K'_{G'_1}$$

therefore,

$$\phi_{\bar{I},2}=K_{G_2} \text{ and } \phi'_{\bar{I},2}=K'_{G_2} \qquad (16)$$

3) The two poisons combine with different active centers exerting "repulsive" influence upon each other. Owing to this mutual action, $K_{G'_1}>K_{G_1}$ and $K'_{G'_1}>K'_{G_1}$, so that

$$\begin{aligned} &K_{G_2}<\phi_{\bar{I},2}<K_{G_2}(1+G_1/K_{G_1}) \\ &K'_{G_2}<\phi'_{\bar{I},2}<K'_{G_2}(1+G_1/K'_{G_1}) \end{aligned} \qquad (17)$$

4) The two poisons combine with different active centers, exerting "attractive" influence upon each other. In this case

$$0 < K_{G'_1}/K_{G_1} < 1 \quad \text{and} \quad 0 < K'_{G'_1}/K'_{G_1} < 1$$

and therefore,

$$0 < \phi_{\bar{1},2} < K_{G_2} \quad \text{and} \quad 0 < \phi'_{\bar{1},2} < K'_{G_2} \qquad (18)$$

The values $K_G$ and $K'_G$ which are equal to $\phi$ and $\phi'$, respectively, have already been determined for each inhibitor. The values $\phi_{\bar{1},2}$ and $\phi'_{\bar{1},2}$ can be determined from the inhibition-pG-curve obtained for $G_2$ in the presence of a definite quantity of another poison $G_1$. By comparing $\phi_{\bar{1},2}$ with $K_{G_2}$ $(=\phi_2)$ and $\phi'_{\bar{1},2}$ with $K'_{G_2}$ $(=\phi'_2)$ we can make clear whether the mutual action of $G_1$ and $G_2$ in their reaction with catalase is "competitive", "independent", "repulsive" or "attractive".

(II) Possible phenomena to be observed by the spectrophotometric method. When a poison $G_1$, capable of changing the spectrum of a catalase, is added to the catalase solution in a large excess, a state will be realized in which practically all catalase molecules are in the form of $EG_1$. If we add to this solution another poison $G_2$ that "competes" with $G_1$ for E, certain number of molecules $EG_1$ will be replaced by $EG_2$, the amount of which being dependent upon the concentration of $G_2$ added. If $G_2$ is added in an extremely large quantity, so that $G_2$ will capture all existing catalase molecule from $G_1$, the solution will eventually show the spectrum of pure $EG_2$, which can be checked by applying $G_2$ alone to the catalase solution.

If, on the other hand, $G_2$ is not competitive with $G_1$, *i*, *e*., if $G_1$ and $G_2$ can combine with E at the same time, the addition of $G_2$ to the pure solution of $EG_1$ will entail transformation of some $EG_1$ molecules into the triple complex $EG_1G_2$, the amount of which being again dependent on the concentration of $G_2$ added. On applying extremely high concentrations of both $G_1$ and $G_2$, we may finally obtain a pure spectrum of $EG_1G_2$, which may differ either from those of $EG_1$ or $EG_2$.

In case $G_1$ and $G_2$ are competitive, we may write

$$\varepsilon = [EG_1] + [EG_2] \quad (19)$$

and, considering Eqs. (1) and (2),

$$\frac{[EG_2]}{[EG_1]} = \frac{K_{G_1} \cdot [G_2]}{K_{G_2} \cdot [G_1]} \quad (20)$$

Let the absorption coefficient of pure $EG_2$ at a certain wave length be $b$, and that of the mixture of $EG_1$ and $EG_2$ at the same wave length be $a$. The ratio $a/b$, which may be desingnated by $\alpha$, can most accurately be determined at the wave lengths where the absorption coefficients of $EG_1$ and $EG_2$ differ most widely. The value $\alpha$ thus determined is equal to the ratio $[EG_1]/\varepsilon$, and therefore,

$$\alpha = \frac{[G_1]}{K_{G_1} \cdot [G_2]/K_{G_2} + [G_1]} \quad (21)$$

By plotting $\alpha$ against $-\log[G_1]$, we may obtain a sigmoid curve of the first order, the middle point of which representing the value $K_{G_1} \cdot [G_2]/K_{G_2}$. As was discussed in our previous paper, the values $K_{G_1}$ and $K_{G_2}$ can be determined separately with each poison. The validity of our theoretical considerations can be checked by comparing the value $K_{G_1} \cdot [G_2]/K_{G_2}$ obtained by the method mentioned above with that calculated from $K_{G_1}$ and $K_{G_2}$.

In case the two poisons are non-competitive, the reaction to occur is

$$EG_2 + G_1 \overset{K_{G'_1}}{\rightleftharpoons} EG_1G_2$$

and the value $\alpha$ determined in the same manner as above will be

$$\alpha = \frac{[EG_1G_2]}{\varepsilon} = \frac{G_1}{K_{G'_1} + G_1} \quad (22)$$

The value $K_{G'_1}$ can be determined from the functional relationship between $\alpha$ and $G_1$, and, as we have discussed already, we can make clear, by the comparison of $K_G$ with $K_{G'}$, whether the mutual action of $G_1$ and $G_2$ is "independent", "repulsive" or "attractive".

*General Results obtained.*

By the application of two methods, kinetic and spectrophotometric, we were able to make it clear that catalase poisons can be divided into four groups, the poisons belonging to the same group acting competitively with each other, and the poisons belonging to different groups acting non-competitively, but either "independently", "repulsively" or "attractively" according to the category of the two poisons combined. These groups will be named, according to their representatives:

(1) Cyanide Type, comprising cyanide, fluoride, chloride and probably sulfide.
(2) Azide Type, comprising azide and hydroxylamine.
(3) Phenol Type, comprising phenol, resorcinol, hydroquinone, cresols, chlorophenols and nitrophenols.
(4) Hydrogen Ion Type, of which hydrogen ion represents the only member thus far found.

Interactions between the substances belonging to these groups are summarized in Table I, where C, R, I and A represent "competitive", "repulsive", "independent" and "attractive" interactions, respectively.

TABLE I.

*Interrelation between two types*

| $G_1$ \ $G_2$ | Cyanide Type | Azide Type | Phenol Type | Hydrogen Ion |
|---|---|---|---|---|
| Cyanide Type | C | R | R | I |
| Azide Type | R | C | R | I |
| Phenol Type | R | R | C | A |
| Hydrogen Ion | I | I | A | C |

Since competitive poisons must be assumed as attaching to the same site on the catalase molecule, while non-competitive ones link to different structural elements, the results obtained lead us to a significant conclusion that there must be at least four sensitive centers in one catalase molecule. Two of these centers having affinities to the substances of cyanide and azide types, respectively, are obviously on the protohemin parts, while the other two assigned to hydrogen ion and the substances of phenol type may presumably be on the protein moiety of the catalase molecule.

To make clear the mutual relationships between four groups mentioned above, discussions must be made for six different cases of combinations, which will considerably complicate our descriptions. In this report we shall restrict ourselves to the discussions of mutual relations between the substances of only three groups, namely, cyanide, azide and phenol groups. The relationship between hydrogen ion and the substances belonging to other groups will be dealt with in our later report.

## Experimental.

(*1*) *Methods.* The techniques employed in this work were virtually the same as those reported previously. In kinetic experiments the "initial" and "final" states of inhibition were sharply distinguished, and for each of these states the degree of inhibition by one poison $G_2$ in the presence of a definite quantity of another poison $G_1$ was measured. The initial state phenomena were studied by the quick method already described, whereby the amount of $H_2O_2$ decomposition was determined about 3 seconds after the addition of $H_2O_2$ to the solution in which thermal equilibrium had been established between E, $G_1$ and $G_2$. To study the final state phenomena, the equilibrium mixture of E, $G_1$ and $G_2$ was first treated with a small quantity of $H_2O_2$ by which E was changed into $\overset{S}{E}$, left at this state for 10 minutes so as to ensure the establishment of equilibrium between $\overset{S}{E}$, $G_1$ and $G_2$, and then larger quantity of $H_2O_2$ was added

and its rate of decomposition was followed. All experiments were performed at pH 7.0 and at 7° and 0°. In all cases, both for "initial" and "final" states, the inhibition-pG-curves were found to represent, in conformity with Eqs. (8) and (13), a typical sigmoid of the first order, and from these curves the values $\phi_{\bar{I},2}$ and $\phi'_{\bar{I},2}$ were determined.

(2) *Interactions between two poisons belonging to the same type.*

(a) Cyanide-type. Combination experiments were carried out at first with cyanide and fluoride, for each of which the following values had been obtained in our previous work:

| | | | |
|---|---|---|---|
| cyanide | initial | $K_G = 10^{-6.2}$ mole/lit. | at 0°C |
| | final | $K'_G = 10^{-6.2}$ mole/lit. | |
| fluoride | initial | $K_G = 10^{-1.9}$ mole/lit. | at 0°C |
| | final | $K'_G = 10^{-2.3}$ mole/lit. | |

The initial inhibition-pG-curve of cyanide in the presence and absence of fluoride (given in concentrations of $10^{-2.0}$ and $10^{-1.5}$ mole/lit.) are shown in Fig. 1 from which it may be seen that the $\phi_{\bar{I},2}$-value is increased by the increase of fluoride concentration present. The value $\phi_{\bar{I},2}$……assuming cyanide as $G_2$ and fluoride as $G_1$…… was found to be $10^{-5.9}$ mole/lit. at $G_1=10^{-2.0}$ mole/lit. and $10^{-5.65}$ mole/lit. at $G_1=10^{-1.5}$ mole/lit. Considering the value $K_G$ given above for fluoride, we obtain from Eq. (15)

$$\phi_{\bar{I},2}=10^{-5.9} \text{ mole/lit. at } G_1=10^{-2.0} \text{ mole/lit.}$$
$$\text{and } \phi_{\bar{I},2}=10^{-5.65} \text{ mole/lit. at } G_1=10^{-1.5} \text{ mole/lit.}$$

The excellent agreement between the calculated and observed values may be taken as an evidence that cyanide and fluoride act competitively with each other.

Similar experiments performed for "final" state showed that

$$\phi'_{\bar{I},2}=10^{-4.9} \text{ mole/lit.} \quad \text{at } G_1=10^{-1.0} \text{ mole/lit.}$$

This figure coincides again satisfactorily with that calculated from Eq. (15). Experiments using cyanide as $G_1$ and fluoride as $G_2$,

on which we shall not enter here for the sake of brevity, also yielded results which were quite in accordance with Eq. (15).

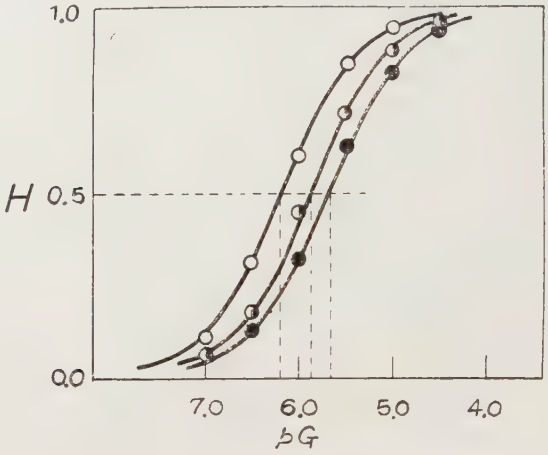


Fig. 1. Effect of fluoride upon the inhibition-pG-curve of cyanide. (Initial state at pH 7.0, 0°).
—○—: KCN alone, $p\phi = 6.2$
—◑—: KCN + $10^{-2.0}$ mole/lit. NaF, $p\phi_{I_2} = 5.9$
—●—: KCN + $10^{-1.5}$ mole/lit. NaF, $p\phi_{I_2} = 5.65$

Further evidence for the competitive interaction between cyanide and fluoride was provided by spectrophotometric experiments. (Cf. Fig. 2. Experimental temperature; 5°). In preliminary experiments it has been confirmed that pure fluoride-catalase spectrum and pure cyanide-catalase spectrum could be obtained by applying to the catalase solution $10^{-1.0}$ mole/lit. sodium fluoride and $10^{-2.0}$ mole/lit. potassium cyanide, respectively. In Fig. 2 the relative values of the spectra ($\Delta \log I_0/I$) shown by the mixture of cyanide- and fluoride-catalase and by the pure cyanide-catalase are plotted against wavelength, taking the absorption coefficient of pure fluoride-catalase as the basis. Considering that at 5° the $K_G$-values for fluoride and cyanide are $10^{-1.8}$ mole/lit. and $10^{-5.8}$ mole/lit. respectively, we may expect from Eq. (20) that at [fluoride] = $10^{-1.0}$ mole/lit. and

[cyanide] $= 10^{-4.0}$ mole/lit. the spectrum to be produced will lie in the middle between those of pure cyanide-catalase and pure fluoride-catalase. This expectation was approximately fulfilled as may be seen from the curves given in Fig. 2.

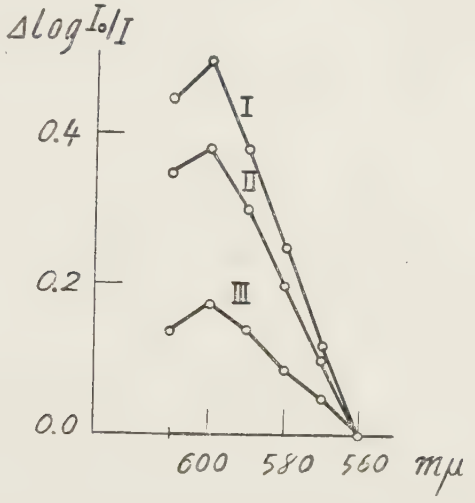


Fig. 2. Effect of cyanide and fluoride upon the spectrum of catalase. (pH 7.0, 5°). Abscissa: NaF $10^{-1.0}$ mole/lit., KCN 0
I: NaF 0, KCN $10^{-2.0}$ mole/lit.
II: NaF $10^{-1.0}$ mole/lit., KCN $10^{-4.0}$ mole/lit.
III: NaF $10^{-1.0}$ mole/lit., KCN $10^{-5.0}$ mole/lit.

With cyanide and chloride, similar kinetic and spectrophotometric experiments as described above were carried out and it was confirmed that these two substances also act competitively with each other.

(b) Azide type. Mutual action between azide and hydroxylamine was studied kinetically, both for "initial" and "final" states, using the two substances alternately as $G_1$ or $G_2$. All the data obtained indicated clearly that these poisons act competitively.

To cite here some results: "initial" states experiments carried out at 7° showed that hydroxylamine, which *per se* gives the $\phi$-

value of $10^{-4.9}$ mole/lit., showed the $\phi_{I,2}$-value of $10^{-4.3}$ mole/lit. when $10^{-5.0}$ mole/lit. of azide was present. For "final" state (at 0°), hydroxylamine *per se* gives $\phi'=10^{-6.9}$ mole/lit., while in the presence of $10^{-6.5}$ mole/lit. Sodium-azide, the corresponding value is shifted to $10^{-5.7}$ mole/lit. (cf. Fig. 3). Calculation from Eq. (15) gives $\phi'_{I,2}=10^{-5.7}$ mole/lit. Competitive action between hydroxylamine and azide was also ascertained by the spectrophotometric method similar to that made with cyanide and fluoride.

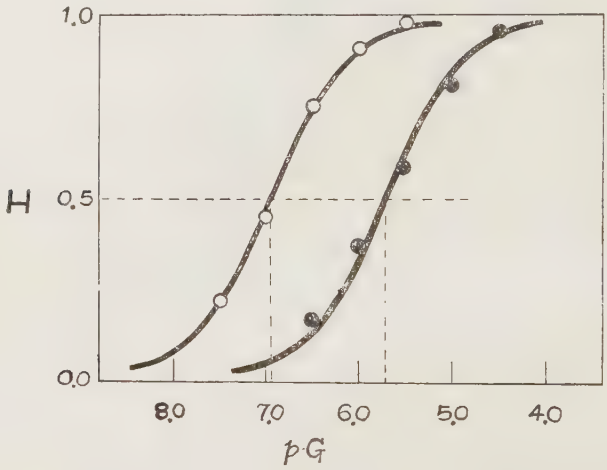


Fig. 3. Effect of azide upon the inhibition-pG-curve of hydroxylamine. (Final state at pH 7.0, 0°).
—○—: $NH_2OH$ alone, $p\phi'=6.9$
—●—: $NH_2OH$ + $10^{-6.5}$ mole/lit. $NaN_3$, $p\phi'_{I,2}=5.7$

(C) Phenol type. For this group we could not carry out the spectrophotometric experiments, because, as was discussed in our previous report, the spectrum of catalase is not modified by the substances of this group. In the kinetic experiments we were able to obtain convincing evidences for their competition in the "final" state. For example, at 0°, *p*-cresol gives the $\phi'_{I,2}$-value of $10^{-3.4}$ mole/lit. in the presence of $10^{-4.0}$ mole/lit. *o*-chlorophenol, while its

*per se* $\phi'$-value is $10^{-4.6}$ mole/lit (cf. Fig. 4). The $\phi'_{I,2}$ value (taking *p*-cresol as $G_2$) calculated from Eq. (15) is $10^{-3.4}$ mole/lit. which agrees satisfactorily with the observed value. Similar kinetic experiments attempted for "initial" state yielded no reliable results,

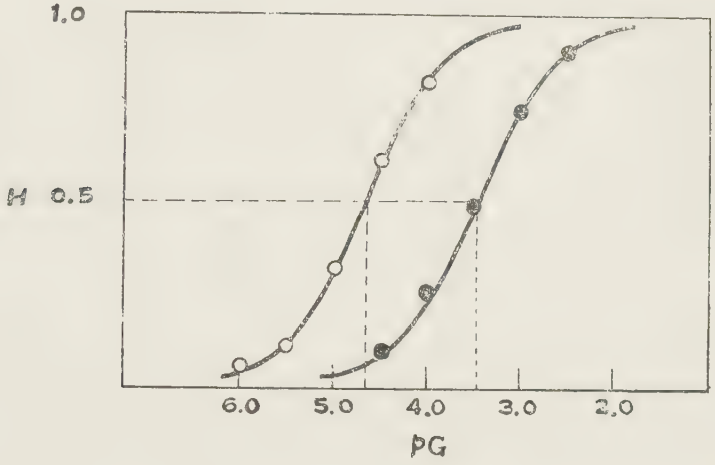


Fig. 4. Effect of *o*-chlorophenol upon the inhibition-pG-curve of *p*-cresol. (Final state at pH 7.0, 0°).
—○—: *p*-cresol alone, $p\phi' = 4.6$
—●—: *p*-cresol + $10^{-4.0}$ mole/lit. *o*-chlorophenol, $p\phi'_{I,2} = 3.45$

since accurate titration of $H_2O_2$ by permanganate was difficult due to the fact that we had to apply a very high concentration of the inhibitors because of their high "initial" $\phi$-values.

Table II. summarizes the representative results obtained in the competitive kinetic experiments carried out with each two poisons belonging to the same group.

TABLE II.

*Kinetic data showing the competition between two poisons belonging to the same group. (Meaning of the notations are in the text.)*

*1. Initial state, pH 7.0.*

| | $G_1$ | $pK_{G_1}$ | $pG_1$ | $G_2$ | $pK_{G_2}$ | $p\phi_{1,2}$ | |
|---|---|---|---|---|---|---|---|
| | | | | | | *Obs.* | *Cal.* |
| Cyanide Type (0°) | NaF | 1.9 | 2.0 | KCN | 6.2 | 5.9 | 5.85 |
| | NaF | 1.9 | 1.5 | KCN | 6.2 | 5.65 | 5.65 |
| Azide Type (7°) | $NaN_3$ | 5.5 | 5.0 | $NH_2OH$ | 4.9 | 4.3 | 4.3 |

*2. Final state, pH 7.0, 0°C.*

| | $G_1$ | $pK'_{G_1}$ | $pG_1$ | $G_2$ | $pK'_{G_2}$ | $p\phi'_{1,2}$ | |
|---|---|---|---|---|---|---|---|
| | | | | | | *Obs.* | *Cal.* |
| Cyanide Type | NaF | 2.3 | 1.0 | KCN | 6.2 | 4.9 | 4.9 |
| Azide Type | $NaN_3$ | 7.7 | 6.5 | $NH_2OH$ | 6.9 | 5.7 | 5.7 |
| | $NaN_3$ | 7.7 | 6.5 | $NH_2OH$ | 6.9 | 5.6 | 5.7 |
| | $NH_2OH$ | 6.9 | 5.5 | $NaN_3$ | 7.7 | 6.2 | 6.3 |
| Phenol Type | *p*-Cresol | 4.6 | 4.0 | *o*-Chloro-phenol | 5.2 | 4.5 | 4.5 |
| | *p*-Cresol | 4.6 | 3.5 | *o*-Chloro-phenol | 5.2 | 4.0 | 4.06 |
| | *o*-Chloro-phenol | 5.2 | 4.0 | *p*-Cresol | 4.6 | 3.4 | 3.4 |

### *(3) Interactions between two poisons belonging to different groups.*

Combination tests of two poisons belonging to different types, namely, Cyanide Type vs. Azide Type, Cyanide Type vs. Phenol Type, and Azide Type vs. Phenol Type, have revealed the fact that in all cases two poisons act mutually "repulsively"; this means that the attachment of one poison to the catalase molecule hinders more or less strongly the attachment of the other poison, though they

occupy different active centers on the surface of the catalase molecule. As may be inferred a priori, distinction between repulsion and competition by kinetic method will become more and more difficult when the repulsive force between two poisons become very strong. This was actually the case in kinetic experiments of combinations of Cyanide Type vs. Azide Type, and of Cyanide Type vs. Phenol Type, but not in the combination of Azide Type vs. Phenol Type. The conclusive evidence of repulsive action between the substances of Cyanide and Azide Types was afforded by spectrophotometric observations, in which the simultaneous attachment of two poisons to one catalase molecule manifested itself in the production of a characteristic new spectra which were different from both the spectra of single poison-catalase-complex formed by each poison.

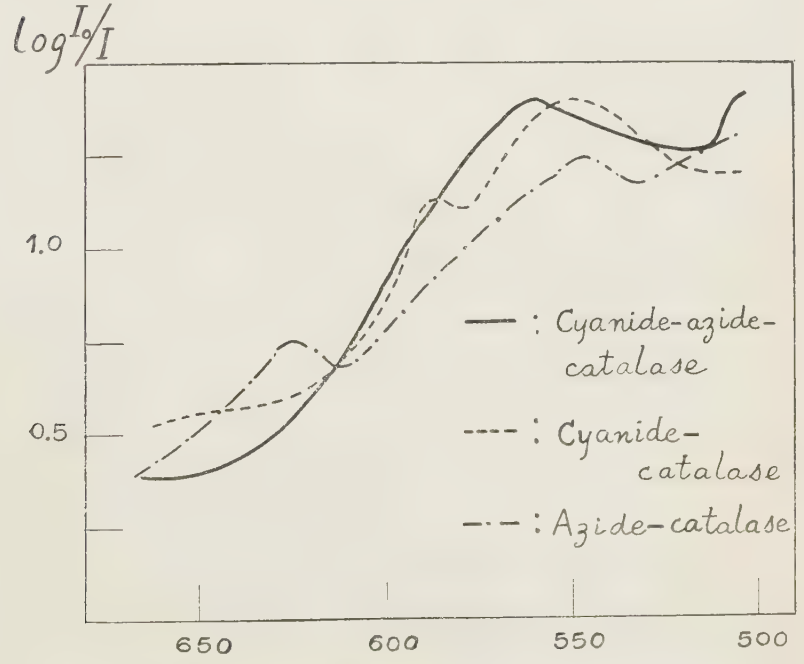


Fig. 5. Spectrum of cyanide-azide-catalase, as compared with those of cyanide- and azide-catalase.

(a) Cyanide Type vs. Azide Type. In an earlier paragraph we have shown that in the presence of sufficiently high concentrations of cyanide and fluoride, catalase solution showed the mixed spectrum of cyanide-catalase and fluoride-catalase, indicating that these two forms were the only catalase-poison-complexes that were formed in that mixture. Quite different phenomenon emerges when a similar experiment is performed with the combination of cyanide and azide, namely, on addition of the two substances in sufficiently high concentrations, catalase solution produces a new spectrum having a maximum at 530 mμ, which is different either from the spectrum of cyanide-catalase or from that of azide-catalase. The spectra of these three complexes are compared in Fig. 5. The new spectrum must be attributed to the formation of cyanide-azide-catalase, both poisons sharing one catalase molecule but attached to different active centers in the protohemin group.

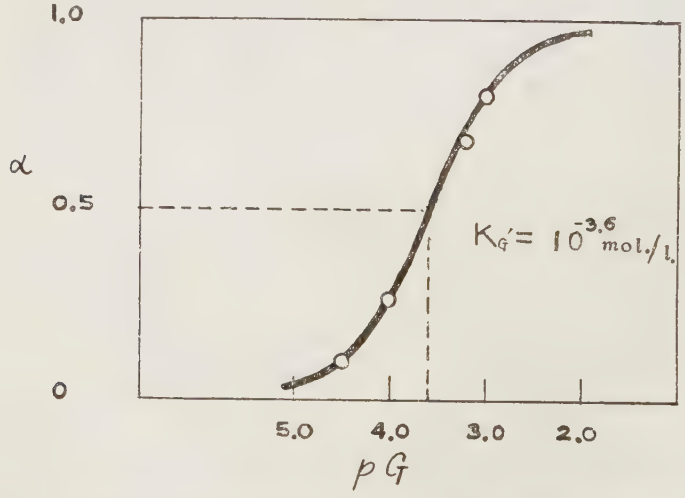


Fig. 6. Spectrophotometric determination of the dissociation constant of the reaction

azide-catalase + cyanide $\underset{K_{G'}}{\rightleftharpoons}$ cyanide-azide-catalase, pH 7.0, 5°.

Ordinate: $\alpha = [\text{cyanide-azide-catalase}]/[\text{total catalase}]$

Abscissa: $-\log [KCN]$ (in the presence of $[NaN_3] = 10^{-2.0}$ mole/lit.).

By adding varying concentrations of cyanide to a "pure" azide-catalase solution (Na-azide added in $10^{-2.0}$ mole/lit.), we measured the functional relationship between $\alpha$-value and cyanide concentration. The results obtained are illustrated in Fig. 6. In conformity with Eq (22) the curve represented a typical sigmoid of the first order. On the basis of Eq. (22) the constant of dissociation of cyanide from the cyanide-azide-catalase was found to be $10^{-3.6}$ mole/lit. at 5°C., a value which is 160 times as large as that of the simple cyanide-catalase-complex. Quite a similar phenomenon was observed when hydroxylamine was used instead of azide. The constant of dissociation of cyanide from the cyanide-hydroxylamine-catalase complex was found to be the same as above, namely $10^{-3.6}$ mole/lit.

Kinetic experiments performed for "initial" state (at 7°) gave the following results: the $\phi$-value of cyanide, which was $10^{-5.7}$ mole/lit. in the absence of other poisons, was found to be $10^{-4.8}$

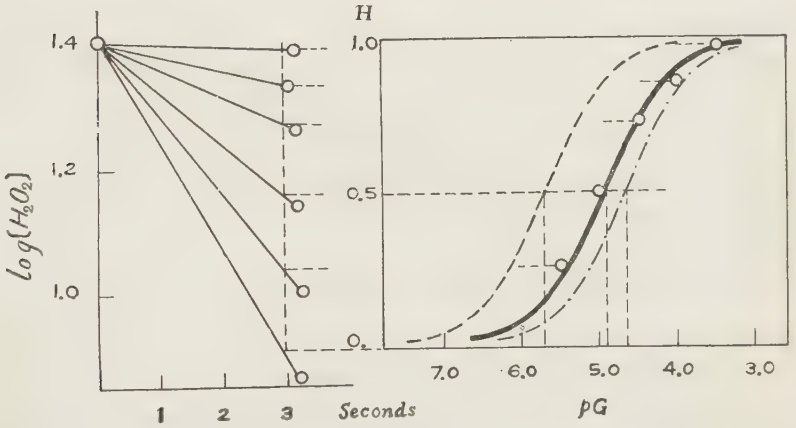


Fig. 7. Inhibition-pG-curve of cyanide in the presence of $10^{-4.5}$ mole/lit. azide. (Initial state at pH 7.0, 7°).

—○—: observed, $p\phi_{\frac{1}{2}}=4.9$.

- - - - -: calculated on the assumption of "independent" interaction. $p\phi_{\frac{1}{2}}=5.7$.

—·—·—: calculated on the assumption of "competitive" interaction. $p\phi_{\frac{1}{2}}=4.65$.

mole/lit. and $10^{-4.9}$ mole/lit. in the presence of $10^{-4.5}$ mole/lit. azide and $10^{-4.0}$ mole/lit. hydroxylamine, respectively. Applying in Eq. (9) the values $K_{G'1}/K_{G1}=160$, $K_{G1}$, $K_{G2}$ and $G_1$, we obtain $\phi_{\bar{1},2}=10^{-4.7}$ mole/lit. and $10^{-4.8}$ mole/lit. which are in good coincidence with the observed values.

For the "final" state it was found that the $\phi'$-value, of cyanide, which *per se* is $10^{-6.2}$ mole/lit., was shifted to $10^{-5.5}$ mole/lit. and $10^{-4.4}$ mole/lit. by the effect of $10^{-7.0}$ mole/lit. azide and of $10^{-5.0}$ mole/lit. hydroxylamine. The corresponding values calculated under the assumption of "competitive" interaction is: $K_{G_2}(1+\frac{G_1}{K_{G_1}})$ $=10^{-5.45}$ mole/lit. and $10^{-4.3}$ mole/lit., respectively, which are equal to the observed values. This coincidence may be due to the fact that in the final state the "repulsive" interaction between cyanide and the poisons of azide type becomes, for some reason unknown at present, stronger so that it can hardly be distinguishable from "competition" by kinetic method. The exact magnitude of this repulsive force could not be determined owing to the fact that the "final" state cannot be investigated by spectrophotometric method.*

For each combination of poisons, we carried out kinetic experiments using alternately, one poison as $G_1$ and the other as $G_2$ and *vice versa*. All the results obtained have indicated that azide and hydroxylamine act repulsively against cyanide both in the "initial" and the "final" state.**

(b) Cyanide Type vs. Phenol Type. The fact that the poisons

* It must be noted that the possibility is not absolutely precluded that the interaction between cyanide and azide might have changed into "competition" as the reaction proceeded to the "final" state. These points must be left open to further investigations.

** From the observations made with microspectroscope, Keilin and Hartree (*2*) concluded that cyanide and azide act competitively. It is conceivable that they were not able to differentiate between the spectrum of cyanide-azide-catalase and that of cyanide-catalase. Our conclusion was substantiated recently by Tanaka (private communication) who showed by magnetometric method that cyanide and azide associate with different protohemins of catalase molecule.

of Phenol Type do not modify the spectrum of catalase, while those of cyanide group do, may be taken as an evidence that the substances of thses two groups attack different active centers in the catalase molecule.

Kinetic experiments carried out with the combination of cyanide vs. *p*-cresol or *o*-chlorophenol showed that at the "final" state, as it was the case with cyanide-azide-combination, two poisons repulse one another so strongly that outwardly it can hardly be distinguished from "competitive" interaction. (See the figures given in Table III.)

This finding is of special theoretical interest in view of our inference that the phenols are attached to the protein moiety of catalase while cyanide combines with protohemin rest. At any rate, it is conceivable that the point of attachment of phenols is located somewhere in the direct vicinity of the structural element to which cyanide is attached. Further evidence that cyanide and phenols attach to different points on the catalase molecule will be adduced in our later report in which it will be shown that the interaction between cyanide and hydrogen ion in their action upon catalase is essentially different from that between phenols and hydrogen ion.*

(c) Azide Type vs. Phenol Type. That the site of attack of phenols is different from that of the substances of azide group is also evident from spectrophotometric data reported in our previous paper. Kinetic experiments using the combination of azide vs. *p*-cresol or *o*-chlorophenol have shown that at the "final" state they act "repulsively", but not so strongly as was the case in the combination with Cyanide Type. For example, *p*-cresol which *per se* gives the $\phi'$-value of $10^{-4.6}$ mole/lit., shows the $\phi'_{\frac{1}{2}}$-value of $10^{-3.2}$ mole/lit. in the presence of $10^{-6.0}$ mole/lit. azide. The $\phi'_{\frac{1}{2}}$-value calculated on the basis of assumption of "competitive" interaction is

* For the reason mentioned already we could not investigate the initial state of interaction between phenols and other poisons.

$10^{-2.9}$ mole/lit. which is decidedly larger than the observed value. The weaker repulsion between the substances of Azide and Phenol Types may be worthy of attention, since the substances of both these types showed very strong repulsive action against substances of the Cyanide Type.

In Table III are summarized the results obtained by the combination experiments carried out with substances belonging to different groups.

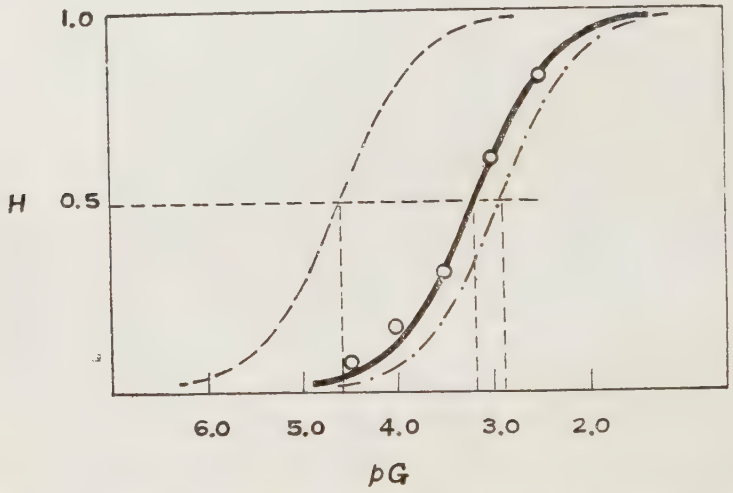


Fig. 8. Inhibition-pG-curve of *p*-cresol in the presence of $10^{-6.0}$ mole/lit. azide (Final state, pH 7.0, 0°).

—○—: observed, $p\phi'_{I,2}=3.2$.

– – – – –: calculated on the assumption of "independent" interaction. $p\phi'_{I,2}=4.6$.

—·—·—: calculated on the assumption of "competitive" interaction. $p\phi'_{I,2}=2.9$.

TABLE III.

*Kinetic data showing interaction between poisons belonging to different groups.*

*1. Initial state, pH. 7.0, 7°.*

| Type | $G_1$ | $pK_{G_1}$ | $pG_1$ | $G_2$ | $pK_{G_2}$ | $p\phi_{\bar{I},2}^{obs.}$ | $p\phi_{\bar{I},2}^{cal.}$ $(K_{G'}/K_G=160)$ | $-\log K_{G_2}\left(1+\frac{K_{G_1}}{G_1}\right)$ |
|---|---|---|---|---|---|---|---|---|
| Cyanide-Azide | $NaN_3$ | 5.5 | 4.5 | KCN | 5.7 | 4.8 | 4.7 | 4.65 |
| | KCN | 5.7 | 5.0 | $NaN_3$ | 5.5 | 4.9 | 4.8 | 4.7 |
| | $NH_2OH$ | 4.9 | 4.0 | KCN | 5.7 | 4.9 | 4.8 | 4.65 |
| | KCN | 5.7 | 5.0 | $NH_2OH$ | 4.9 | 4.1 | 4.1 | 4.0 |

*2. Final state, pH. 7.0, 0°C.*

| Type | $G_1$ | $pK'_{G_1}$ | $pG_1$ | $G_2$ | $pK'_{G_2}$ | $p\phi_{\bar{I},2}^{obs.}$ | $-\log K'_{G_2}\left(1+\frac{K'_{G_1}}{G_1}\right)$ |
|---|---|---|---|---|---|---|---|
| Cyanide-Azide | KCN | 6.2 | 5.0 | $NaN_3$ | 7.7 | 6.5 | 6.5 |
| | KCN | 6.2 | 5.0 | $NH_2OH$ | 6.9 | 5.5 | 5.6 |
| | $NH_2OH$ | 6.9 | 5.0 | KCN | 6.2 | 4.4 | 4.3 |
| | $NaN_3$ | 7.7 | 7.0 | KCN | 6.2 | 5.5 | 5.45 |

| Type | $G_1$ | $pK'_{G1}$ | $pG_1$ | $G_2$ | $pK'_{G2}$ | $p\phi^{obs.}_{1'2}$ | $-\log K'_{G2}\left(1+\frac{G_1}{K'_{G1}}\right)$ |
|---|---|---|---|---|---|---|---|
| Cyanide-Phenol | *p*-cresol | 4.6 | 4.0 | KCN | 6.2 | 5.5 | 5.6 |
| | KCN | 6.2 | 5.5 | *p*-cresol | 4.6 | 3.7 | 3.8 |
| | KCN | 6.2 | 5.0 | *o*-chlorophenol | 5.2 | 4.0 | 4.0 |
| | NaF | 2.3 | 2.0 | *o*-chlorophenol | 5.2 | 4.7 | 4.7 |
| Azide-Phenol | $NaN_3$ | 7.7 | 6.0 | *p*-cresol | 4.6 | 3.2 | 2.9 |
| | *p*-cresol | 5.2 | 4.0 | $NaN_3$ | 7.7 | 6.9 | 6.5 |
| | $NaN_3$ | 7.7 | 6.5 | *o*-chlorophonol | 5.2 | 4.3 | 4.0 |

(3) Phenomena to be observed at non-equilibrium state. The phenomena dealt with hitherto were all concerned with the equilibrium state established between catalase molecule (E or $\overset{S}{E}$) and one or two different poisons. Under certain conditions we may happen to observe phenomena at non-equilibrium state which are more or less different from those described above. The most salient cases occur in the combination experiments using cyanide, on the one hand, and the substances of azide and phenol type on the other.

As has been noticed in our previous paper, the equilibrium between cyanide and catalase molecule is always attained very rapidly, while the substances of Azide and Phenol Type react rather slowly with the catalase. Let us denote cyanide by $G_1$ and some substance of Azide or Phenol Type by $G_2$, and consider their reaction with $\overset{S}{E}$ which can be formed immediately after addition of $H_2O_2$ to E. When $G_1$ and $G_2$ are simultaneously added to E, or when $G_1$ alone is added at first to E and then $G_2$ introduced, the first event to occur is the establishment of equilibrium between $G_1$ and the whole $\overset{S}{E}$ present, and the decomposition of $H_2O_2$ by $\overset{S}{E}$ whose concentration is determined by the said equilibrium:

$$G_1 + \overset{S}{E} \rightleftharpoons \overset{S}{E}G_1 \qquad (23)$$

If, therefore, the rate of decomposition of $H_2O_2$ is followed from the very moment at which $G_2$ was added, we can at first observe only the inhibition caused by $G_1$; thus outwardly $G_2$ appears to exert no influence upon the action of $G_1$. Gradually, however, $G_2$ reacts with the existing $\overset{S}{E}$, causing the shift of equilibrium of (23), which eventually lead to the state in which $G_1$ and $G_2$ appear to behave mutually repulsively.

Similar events occur when $G_2$ and $\overset{S}{E}$ are brought together beforehand and allowed to attain an equilibrium

$$G_2 + \overset{S}{E} \rightleftharpoons \overset{S}{E}G_2 \qquad (24)$$

and then $G_1$ added. In this case, $G_1$ reacts with the existing $\overset{S}{E}$

attaining instantaneously the equilibrium demanded by (23). Consequently, the concentration of $\overset{S}{E}$ will fall off to the value which no longer corresponds to the equilibrium concentration demanded by (24). However, owing to the sluggishness of the reaction (24)— which implies the rigidness in the shift of the equilibrium— a certain time must elapse before the new equilibrium between $G_1$, $G_2$ and $\overset{S}{E}$ is accomplished. For this reason, if the rate of decomposition of $H_2O_2$ is traced from the very moment at which $G_1$ was added, the poisons $G_1$ and $G_2$ will appear to behave at first mutually independently till the preexisting rigid equilibrium (21) is dissolved into the true new equilibrium.

Mutual actions between different poisons as they were discussed in the preceeding paragraphs were all studied under the condition in which such transient phenomena as described above could not come into question.

## Summary.

1) Interactions between two poisons in their action upon catalase were studied both by kinetic and spectrophotometric methods. Based on the results obtained, poisons of catalase were divided into four types, *i. e.* Cyanide, Azide, Phenol and Hydrogen Ion Types. To each of these groups belong the following substances:

Cyanide Type: cyanide, chloride, fluoride and sulfide.

Azide Type: azide and hydroxylamine.

Phenol Type: phenol, resorcinol, hydroquinone, cresols, chlorophenols and nitrophenols.

Hydrogen Ion Type, of which hydrogen ion represents the only member thus far found.

The study of the mutual action of the substances belonging to the first mentioned three groups formed the subject of the present report.

2) Poisons belonging to the same group "compete" with each other for a definite active center of the catalase molecule; accord-

ingly, two poisons, $G_1$ and $G_2$, belonging to the same group cannot share one catalase molecule (E) at the same time. This was shown, not only by kinetic method but also spectrophotometrically—in the case of substances of Cyanide and Azide Types—by the fact that on adding $G_1$ and $G_2$ in sufficient quantities, catalase solution shows only the mixed spectra of $EG_1$ and $EG_2$, the ratio of which depends upon the relative concentrations of $G_1$ and $G_2$ and their relative affinities toward E.

3) Poisons belonging to different groups are linked to different active centers in the catalase molecule, a conclusion which has partly been drawn in our previous paper in which we inferred that the poisons of Phenol Type attach to the protein moiety, while those of Cyanide and Azide Types combine with protohemins of the catalase molecule. Owing to the difference of the site of attachment, poisons belonging to different groups may be expected to link to one catalase molecule at the same time, forming a triple complex of the type $EG_1G_2$. This expectation was borne out not only by various kinetic experiments, but also—for the substances of Cyanide and Azide Types—spectrophotometrically by the discovery of a characteristic spectrum of $EG_1G_2$ which was essentially different from either that of $EG_1$ or of $EG_2$.

4) By analyses of various quantitative data, it was shown that the affinity of $G_1$ to $EG_2$ is not always the same as that between $G_1$ and E; in other words, poisons belonging to different types can exert certain mutual influences in their combination with catalase molecule. The substances of the three categories dealt with in this paper were found to act upon each other more or less "repulsively", *i. e.* $G_1$ requires more energy to combine with $EG_2$ than to combine with E. The data to be reported later will show that the hydrogen ion, when attached to catalase molecule, act "attractively" on phenols, but quite "indifferently" upon substances belonging to the Cyanide and Azide Types.

5) On the available data it may be concluded that the catalase

molecule has at least four active or sensitive centers, each having affinities to the poisons or respective types mentioned above.

This investigation forms a part of study of the Research Commitee of Heavy Metal Catalysis of the National Research Council and was supported by a grant donated to one of the authers (Tamiya) by the Ministry of Education.

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(1) Ogura, Y., Tonomura, Y., Hino, S. and Tamiya, H., J. Biochem. **37**, 153 (1950).
(2) Keilin, D. and Hartree, E. F., Proc. Roy. Soc. London, B, **121**, 173 (1937).

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# ON THE TUBERCULOSTATIC ACTIONS OF VARIOUS ORGANIC COMPOUNDS.

## I. SULFA COMPOUNDS.

By

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(Received for Publication on March 1, 1950)

Since RICH & FOLLIS (1938) have tried sulfanilamide in the experimental tuberculosis in guinea-pigs, various sulfa compounds were studied on these effects against the acid-fast bacilli in vitro as well as in vivo. Among them, Promin, Promizole, Diasone, and Sulphetrone were found promising in clinical trials. But, unfortunately, they showed some toxic effect upon the organisms in the course of long-run therapy.

So, it is desirable, if possible, to find out any new compounds which are sufficiently active and at the same times even in long-continued application. For this purpose, the author tried, as first approach, a series of assay in vitro with various sulfa compounds* against a strain of avian type tubercle bacilli, and for some of the most effective ones, the toxicity was examined against organisms using mice as test animal.

### EXPERIMENTAL.

1. Methods for in vitro assays.—The test compounds are dissolved in any good solvent such as redistilled water, acetone, dilute alcohol, or glycerin, in the concentration of 10 mg. per 10 ml. From this solution, a serial dilution was made with redistilled water and then

* The author expresses here his profound thanks for the kind presentations of new compounds from Prof. M. ISHIDATE and his colleagues and the YOSHITOMI Drug Manufacturing & Co., Ltd.

0.3 ml of each diluted solution were added to 2.7 ml of the culture medium. Final concentration of the compound in each test tube will be shown in Table I.

TABLE I.

| No. of test tubes | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Final conc. (mg. %) | — | — | 1 | 0.5 | 0.2 | 0.1 | 0.05 | 0.02 | 0.01 | 0.005 | 0.002 | 0.001 |
| (in the case of weak cmpd.) | 10 | 5 | 2.5 | 1.25 | 0.63 | 0.31 | 0.16 | 0.08 | 0.04 | 0.02 | 0.01 | 0.005 |

When an organic solvent was used, its effect on the culture was observed as control. Caution were taken also for the compounds which are thermolabil or acid-unstable not to destroy them by heat sterilization or acidification.

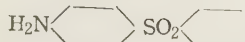
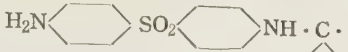
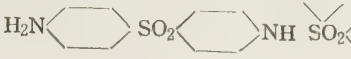
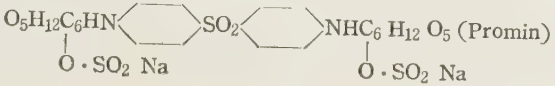
As culture media, the following BLOCH's modification of the LOCKEMAN's solution[1] is used:

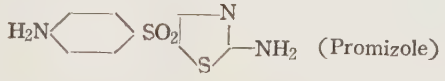
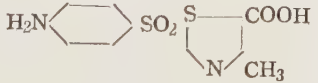
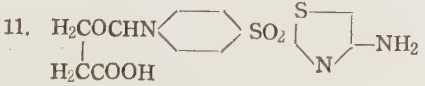
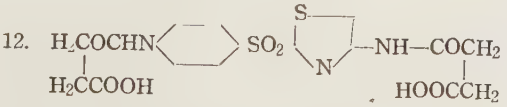
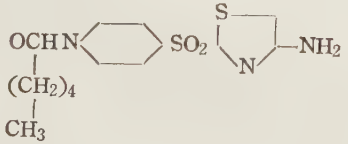
| | |
|---|---|
| Sodium monophosphate | 1.5 g. |
| Potassium diphosphate | 2.0 g. |
| Magnesium sulfate | 1.25 g. |
| Sodium citrate | 1.25 g. |
| Mohr's salt | 0.005 g. |
| Glycine | 2.5 g. |
| Glycerine | 12.5 ml. |
| Aq. redistillata | 500.0 ml. |

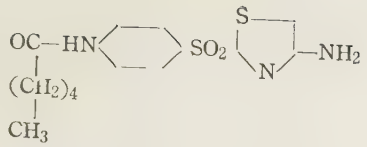
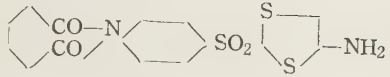
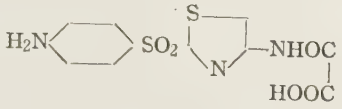
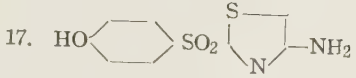
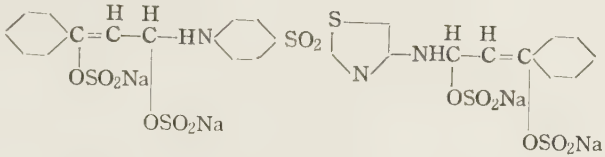
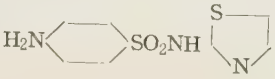
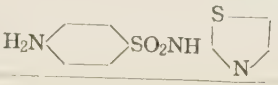
The medium, after neutralyzed with *N*-NaOH and boiled a short time, was filtered and autoclaved.

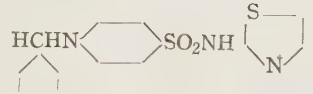
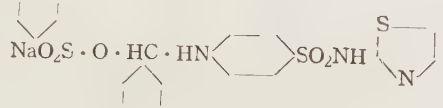
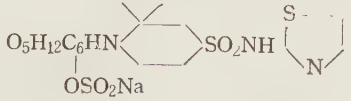
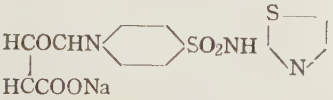
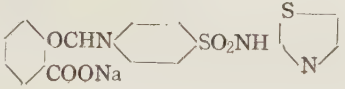
The test microorganism, cultivated on glycerin-agar slant during a week, was harvested and weighed; then, thoroughly mixed with culture media to become 1% suspension. The amount of bacteria inoculated was about 0.4 mg. for each tube.

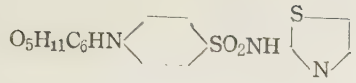
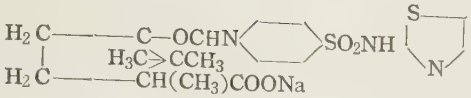
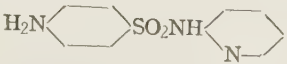
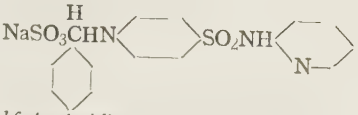
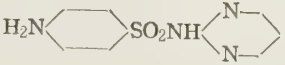
TABLE II.

| Compounds | Molecular Weight | Minimum Bacteriostatic concentration (mg. %) | | | Index of Comparative effect % 7th day | $LD_{50}$ for mice (mg.) |
|---|---|---|---|---|---|---|
| | | 3rd day | 5th day | 7th day | | |
| *I. Diphenyl sulfones* | | | | | | |
| 1.  (DDS) | 248 | 0.05 | 0.1 | 0.1 | 100 | 30[2] |
| 2.  | 290 | — | >1 | >1 | <12 | |
| 3.  | 440 | — | 0.5 | 1 | 18 | |
| 4.  | 482 | 1 | 1 | >1 | <10 | |
| 5.  (Promin) | 780 | 0.1 | 0.2 | 0.2 | 157 | 65[3] |
| 6.  | 249 | 0.08 | 0.16 | 0.31 | 32 | |
| 7.  | 265 | 0.04 | 0.31 | 0.63 | 17 | |
| 8.  | 264 | 0.08 | 0.31 | 0.31 | 34 | |

| Compounds | Molecular Weight | Minimum Bacteriostatic concentration (mg. %) 3rd day | 5th day | 5th day | Index of Comparative effect % 7th day | $LD_{50}$ for mice (mg.) |
|---|---|---|---|---|---|---|
| *II. Promizole and its derivatives.* | | | | | | |
| 9.  (Promizole) | 255 | 0.05 | 0.1 | 0.5 | 21 | |
| 10.  | 274 | — | >10 | >10 | <1 | |
| 11.  | 355 | 2.5 | 5 | 10 | 1 | |
| 12.  | 441 | 10 | 10 | >10 | <2 | |
| 13.  | 353 | 5 | 10 | >10 | <1 | |

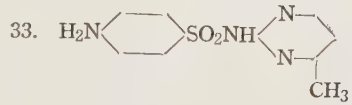
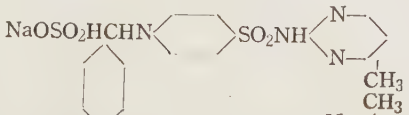
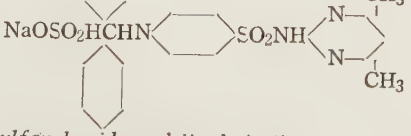
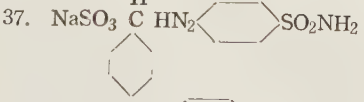
| | | | | | | |
|---|---|---|---|---|---|---|
| 14.  | | 381 | 2.5 | 5 | 10 | 2 |
| 15.  | | 385 | 2.5 | 5 | 10 | 2 |
| 16.  | | 403 | 1.25 | 2.5 | 5 | 3 |
| 17.  | | 256 | 10 | 10 | <10 | <1 |
| 18.  | | 895 | 2.5 | 5 | 10 | 4 |
| *III. Sulfathiazole and its derivatives.* | | | | | | |
| 19.  | (S T) | 255 | 0.02 | 0.02 | 0.02 | 520 | 15-20 |
| 20.  | (S M T) | 269 | 0.02 | 0.05 | 0.2 | 53 | 15[2] |

| Compounds | Molecular Weight | Minimum Bacteriostatic concentration (mg. %) | | | Index of Comparative effect % 7th day | $LD_{50}$ for mice (mg.) |
|---|---|---|---|---|---|---|
| | | 3rd day | 5th day | 7th day | | |
| 21.  | 345 | >1 | >1 | >1 | <14 | |
| 22.  | 447 | 0.1 | 0.1 | 0.1 | 180 | >50 |
| 23.  (STGS) | 521 | 0.02 | 0.05 | 0.05 | 420 | >100 |
| 24.  | 377 | 10 | 10 | 10 | 2 | |
| 25.  | 375 | >1 | >1 | >1 | <15 | |
| 26.  | 425 | 0.2 | 0.2 | 0.2 | 84 | >40 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 27.  | (STG I)* | 417 | 1.0 | 0.2 | 0.2 | 84 | >100 |
| 28. ibid. | (STG II)** | | 0.5 | 0.1 | 0.2 | | |
| 29.  | | 445 | >1 | >1 | >1 | <18 | |
| *IV. Sulfapyridine and its derivatives.* | | | | | | | |
| 30.  | (S P) | 249 | 0.5 | 0.5 | 0.5 | 20 | 10-15[2] |
| 31.  | | 441 | >1 | >1 | >1 | <21 | |
| *V. Sulfapyrimidine and its derivatives.* | | | | | | | |
| 32.  | (S D) | 250 | 0.1 | 0.5 | 0.5 | 20 | |

* Water soluble without heating.

** Water insoluble without heating.

| Compounds | Molecular Weight | Minimum Bacteriostatic concentration (mg. %) 3rd day | 5th day | 7th day | Index of Comparative effect % 7th day | $LD_{50}$ for mice (mg.) |
|---|---|---|---|---|---|---|
| 33.  (S M D) | 265 | 0.05 | 0.2 | 0.2 | 55 | |
| 34.  | 456 | 1 | >1 | >1 | <18 | |
| 35.  | 470 | >1 | >1 | >1 | <19 | 100[2)] |
| *VI. Sulfanylamide and its derivatives.* | | | | | | |
| 36.  (S A) | 172 | 0.5 | 10 | 10 | 1 | |
| 37.  | 364 | 0.5 | 10 | 10 | 1 | |
| 38.  | 252 | >10 | >10 | >10 | <1 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 39.  | 362 | — | 0.31 | 0.31 | 5 | |
| *VII. Miscellaneous* | | | | | | |
| 40.  | 264 | 0.2 | 0.5 | 0.5 | 20 | 302) |
| 41.  | 530 | 2 | 10 | 10 | 2 | |
| 42.  | 796 | 5 | 10 | 10 | 3 | |
| 43.  | 213 | 5 | 10 | 10 | 1 | |
| 44.  | 263 | 5 | 10 | >10 | 1 | |
| 45.  | 319 | 5 | 5 | 10 | 1 | |
| 46.  | 298 | 10 | 10 | >10 | 1 | |
| 47.  | 313 | >10 | >10 | >10 | <1 | |
| 48.  | 264 | 5 | >10 | >10 | <1 | |

At 37°, after 2 days of lag periods, the microorganism in the control medium showed the growth on the surface or in the bottom of medium, and seemed to finish their logarithmic phase of growth at the end of 6th day.

The results of effectiveness are, therefore, judged after 7 days. incubation by the state of surface growth. For the convenience, the growth was always compared to the parallel experiments using diamino diphenyl sulfone and expressed in arbitrary units putting the bacteriostatic action of this medicament as 100 %. The results are summarized in Table II.

2. Methods for toxicity tests.—The test mice, weighing about 15g, were used after 2 weeks feeding at a definite condition in the laboratory. Then 4 mice in each lot were observed during 10 days, after 0.5 ml. subcutaneuous injections of test compounds.

The results are shown in Table III.

TABLE III. *Toxicity test for 15g. mice*

| No. of Cmpd. / Dosis | 22 Benzyl ST sulfit-Na | 23 STGS | 25 Phthalyl ST | 28 STG II | 19 ST (Na-salt) |
|---|---|---|---|---|---|
| 100 mg | — | 0/4 | — | 0/4 | — |
| 50 | 0/4 | 0/4 | — | 0/4 | 4/4 |
| 40 | — | — | 0/4 | — | — |
| 25 | — | 0/4 | — | 0/4 | 4/4 |
| 20 | 0/4 | 0/4 | 0/4 | — | — |
| 15 | 0/4 | — | — | — | — |
| 10 | 0/4 | — | 0/4 | — | 0/4 |
| 5 | 0/4 | — | — | — | — |
| Control (Saline 0.5 ml) | 0/4 | 0/4 | 1/4 | — | 0/4 |

The number represents *The ratio of dead animals over survived animals.*

## Discussion.

In the diphenyl sulfone series, the most powerful tuberculostatic agent is diamino diphenyl sulfone (D.D.S.) (*1*); but from the view point of toxicity, the most promising compound may be Promin (*5*).

Glucose sulfit-Na or benzyl sulfit-Na are also worthy of consideration being less toxic.

In the groups of Promizole and its derivatives, the most effective one is Promizole (*9*). The other compounds show only slight bacteriostatic action.

Among the derivatives of Sulfathiazole, there are many effective compounds: Sulfathiazol (ST) (*19*) is most effective and Sulfathiazole glucose sulfit- Na (STGS) (*23*) the next, but the toxicity of the latter compound is greatly reduced than Sulfathiazol: the dosis of 100 mg for 15 g mouse showing no noticeable toxicity after a subcutaneous injection. Besides STGS, Sodium salt of Phthalyl sulfathiazol (*24*), Sufathiazol glucoside I & II (STG) (*27*, *28*) and Sulfathiazole benzyl Sulfit-Na have also the greater tolerance for mice without much loss of the original bacteriostatic action of ST. However, in the same series of $N^4$-substituted Sulfathiazole, benzyl, (*21*) succinyl (*24*), malenyl (*25*) and camphosyl Sulfathiazol (*28*) have only a poor bacteriostatic action.

The antibacterial effects of lesser degree are seen in thh series of the Sulfapyridine, Sulfapyrimidine and Sulfanylemide except the compounds No. *33*. (Sulfa monomethyl pyrimidine) which has about half of the DDS's potency.

Yet, some authors (4) (5) (6) (7) postulate that in the series of sulfa compounds, Sulfathiazole and diphenyl sulfones are equally effective in vitro test; but in the case of clinical or experimental tuberculosis, Sulfathiazole is far more inferior to diphenyl sulfones.

In this regard Sasano (7) observed that Promin has a bacteriocidal effect while Sulfathiazole bacteriostatic. But it can not be concluded from this observation that any drugs which act bacteri-

ocidally is more effective in the treatment of tuberculosis than those which act bacteriostatically, because Streptomycin has only bacteriostatic action in clinical dosis.

Probably one of the reason why Sulfathiazole is not active in vivo experiment is due to its high toxicity when sufficient dosis is administered. Relatively higher effectiveness of STGS or STG can be also presumed by their less toxicity. As the cause of lower toxicity of STGS, Mashimo (8) has claimed that it might be due to the fact that STGS is practically not absorbed on the surface of red blood cells, while the other sulfa drugs are more or less absorbed.

According to this experiment, the order of absorbtion is as follows, which stand in good agreement with the toxicity.

$$SA > SP > ST > SD > STG > STGS$$

The excretion rate of STGS into urine is also very high when administered intravenously to rabitts. By this fact, some one would assume that STGS is so quickly excreted in the urine that it might be unable to yield a sufficient concentration of ST in the bodies to which owe the bacteriostatic action of STGS. But this is not true since STGS showed succesful result in the treatment of Ekiri (10) as well as experimental streptococcal infection in mice. (9)

In this line the reports of Sweany (5) and Duca & Steinbach (10) are worthy of mention. They showed a quite concordant result with this report, that DDS, Promin, SP and SA are effective in vitro tests against various acid-fast bacilli.

## Conclusion.

The author compared the effectiveness of forty-eight sulfa compounds against acid fast bacilli in vitro. Some of them were tested on the acute toxicity for mice. The most effective compounds were found in the group of diamino diphenyl sulfones and Sulfathiazole. Among them, Sulfathiazole glucose sulfit-Na is most promising on account of less toxicity.

The author is most grateful to Prof. K. KODAMA and Prof. M. ISHIDATE for their helpful advice; and to Miss Y. TOYOIZUMI and Miss R. KITAMURA for their technical assistance.

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# ON THE QUANTITATIVE INTERPRETATION OF ELECTROPHORESIS EXPERIMENTS WITH TISELIUS APPARATUS.

By

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## *I. Introduction.*

Since Schlieren method was first used by Tiselius in the electrophoresis of proteins, it has become one of the most powerful methods for the research of blood plasma proteins. Its optical system was afterwards improved by Svensson (1), Philpot (2), and Longsworth (3) (Schlieren diagonal method and Schlieren scanning method), and many useful results were obtained in the feild of biological, colloidal and immunological chemistry. In this concern, the theoretical consideration on the moving boundaries of strong electrolytes was fully discussed by Dole in 1945 (4), which was justified by the experiments performed by Longsworth (5). His theory clarified, if not all, many points of ambiguity which attached to the explanation given to various phenomena such as, for example, δ-and ε-boundaries or mobility differences between ascending and discending boundaries.

As far as I know, none has attempted to apply his theory to calculate true mobility and true percentage of each component from experimental results of electrophoresis of plasma proteins.

There are assumptions made in the Dole's theory which seem plausible for dilute solution of strong electrolytes. These assumptions seem to be also plausible for proteins as a first approximation. In this report the results of the application are given.

## *II. Dole's theory of moving boundaries.*

For simplicity, I have listed the results of Dole's theory, applied to the case of electrophoresis of blood plasma in Table I, II, III, IV and Fig. 1, 2. Notations used in these figures and tables are the same as those used in the Dole's paper (4), except that quantities of discending boundaries are primed and that non-primed quantities are those of ascending boundaries. For example $V^{\mu\nu\prime}$ and $c_i{}^{\mu\prime}$ are velocity of the boundary between $\mu$ and $\nu$ phase and concentration of the $i$-th component in $\mu$ phase in the discending boundary respectively.

Notations are as follows;

$r_j$; relative mobility of the $j$-th species

$\sigma^\nu$; relative conductance of the $\nu$ phase

$V^{\mu\nu}$; velocity of the boundary between the $\mu$ and $\nu$ phase under unit current

$cj^\nu$; equivalent volume concentration of the $j$-th species in the $\nu$ phase. Greek letters indicate phases. Number suffix indicates the species except in the case of $y_j$'s.

## *III. Calculation of true mobilities.*

First of all, we can determine true mobilities of all the components from Table I and III. Of many quantities listed in these Tables what can be known from experiments are all the $V^{\mu\nu\prime}$'s, $\sigma^\alpha$, $\sigma^{\eta\prime}$, $\sigma^{\alpha\prime}$ and $\sigma^\eta$. Inserting the experimental values of $V^{\alpha\beta\prime}$, $\sigma^{\alpha\prime}$ and $V^{\alpha\beta}$, $\sigma^\alpha$ in equations (13) and (1) respectively, we obtain $r_6$ and $y_5$. We further insert $r_6$ thus obtained and $V^{\beta\gamma}$ in eq. (7) to obtain $\sigma^\beta$. Thus use of this $\sigma^\beta$ and $V^{\beta\gamma}$ in eq. (2) yields the value of $y_4$. In the same way we can obtain $\sigma_\beta{}'$ and $r_5$ from $y_5$ eq. (19) and eq. (14). Repeating analogous calculations we can obtain all the values of $y_i$'s, $r_i$'s and $\sigma^\nu$'s. These mobilities are relative mobilities in the arbitray unit, and in the above calculation we can use displacement of each component measured on the photographic plate as $V^{\mu\nu}$'s and

$V^{\mu\nu\prime}$'s. If we want to know the absolute values, the measurements of absolute values of electric current, conductivity, time of electrophoresis, boundary displacements and cross-sectional area of electrophoresis cell are necessary. From above quantities we can obtain the absolute value of the mobility of albumin from the discending boundaries, and those of other components are obtained at once from relative mobilities.

### *IV. Percentage of each component.*

Since we have obtained the values of $y_i$'s and $r_i$'s in the above section, we can use these values in Table II and IV to calculate the concentration change of each component in each boundary. $c_j$'s are equivalent volume concentrations, and of cource, these are proportional to the concentration in gm./dl., viz. $c_j=k_jC_j$; where $C_j$ is the concentration of the $j$-th component in gm./dl. and $k_j$ is a proportianality constant. For simplicity we make here an approximation that the changes of concentrations of buffer ions in each boundary are small and the contribution of these ions to the area of each boundary can be neglected. This assumption can be allowed, for $r_j$'s of the buffer ions are large compared with those of proteins. Further, if we take refractive index increment of all the components equal, areas of peaks of the pattern are as follows:

$$\begin{aligned}
A_6 &= C_6{}^{\beta} \\
A_5 &= C_6{}^{\gamma} - C_6{}^{\beta} + C^{\gamma} \\
A_4 &= C_6{}^{\delta} - C_6{}^{\gamma} + C_3{}^{\delta} - C_5{}^{\gamma} + C_4{}^{\delta} \\
A_3 &= C_o{}^{\varepsilon} - C_6{}^{\delta} + C_5{}^{\varepsilon} - C_5{}^{\delta} + C_4{}^{\varepsilon} - C_4{}^{\delta} + C_3{}^{\varepsilon} \\
A_2 &= C_6{}^{\zeta} - C_6{}^{\varepsilon} + C_5{}^{\zeta} - C_5{}^{\varepsilon} + C_4{}^{\zeta} - C_4{}^{\varepsilon} + C_3{}^{\zeta} - C_3{}^{\varepsilon} + C_2{}^{\zeta}
\end{aligned} \tag{25}$$

$$\begin{aligned}
A_6' &= C_6{}^{\alpha\prime} - C_5{}^{\beta\prime} + C_5{}^{\alpha\prime} - C_4{}^{\beta\prime} + C_4{}^{\alpha\prime} - C_3{}^{\beta\prime} + C_3{}^{\alpha\prime} - C_2{}^{\beta\prime} + C_2{}^{\alpha\prime} \\
A_5' &= C_5{}^{\beta\prime} - C_4{}^{\gamma\prime} + C_4{}^{\beta\prime} - C_3{}^{\gamma\prime} + C_3{}^{\beta\prime} - C_2{}^{\gamma\prime} + C_2{}^{\beta\prime} \\
A_4' &= C_4{}^{\gamma\prime} - C_3{}^{\delta\prime} + C_3{}^{\gamma\prime} - C_2{}^{\delta\prime} + C_2{}^{\gamma\prime} \\
A_3' &= C_3{}^{\delta\prime} - C_2{}^{\varepsilon\prime} + C_2{}^{\delta\prime} \\
A_2' &= C_2{}^{\varepsilon\prime}
\end{aligned} \tag{26}$$

percentage of the $j$-th component

$$= 100 \times C_j / \Sigma C_j \qquad (27)$$

From Table II, IV, eqs. (25) and (26), we obtain two sets of percentages of all the components. These two sets of values should agree, if Dole's theory is applicable for our case. Experimental results show that this is the case (Table V) proving our assumption is justified.

### *V. Experimental and the results.*

Three section type Tiselius electrophoresis cell was used. The optical system was Schlieren diagonal methed. Human plasma (citrated plasma) was diluted about three times and was dialysed against phosphate buffer (ionic strength 0,18, pH 7,9) for 24 hours in cellophane bag. Current stabilizing circuit was used, current being constant during the experiment. Conductivity measurement was perfomed at the temperature of electrophoresis, which was between 6,0°C and 10,0°C. Photograph was taken after about 1.5 hours under the current of 18,0 mA. Measurement of boundary displacement was obtained from the distance between the highest position of $\delta$-or $\epsilon$-boundary and that of the peak of each component. When the shape of the peak was far from symmetrical, position of the first moment of that peak was used instead of the highest position. Experimental results are listed in Table V.

### *VI. e/M of the protein particle.*

Dole's theory teaches us that the transport number of every component can be calculated from $r_i$'s and $y_i$'s. While, the transport number is by its definition

$$T_j = \frac{c_j r_j}{\Sigma c_j r_j} = \frac{\frac{C_j}{M_j} e_j r_j}{\Sigma \frac{C_j}{M_j} e_j r_j} \qquad (j=1,\ldots, 7)$$

where

$e_j$ ; charge per particle of the $j$-th component

$c_j$ ; equivalent volume concentration of the $j$-th component

$C_j$ ; concentration in gm./dl. of the $j$-th component
$M_j$ ; molecular weight of the $j$-th component
$r_j$ ; mobility of the $j$-th component

From these equations we can calculate the important property $e/M$ of the particle. Of course, for this calculation many experimental quantities should be worked out, so that the results will be expected to be somewhat inexact. The results of this calculation will be published later from our laboratory.

### *VII. Some remarks on the interpretation of the experimental data of the electrophoresis experiments.*

Our results show shat apparent percentage of albumin is always a few percent larger, and that of $\gamma$-globulin is always a few percent smaller that their true values, while true values of $\alpha$-, $\beta$-globulin and fibrinogen are in some cases larger and in other cases smaller than the apparent values. These results show that in the interpretation of the results of electrophoresis of blood plasma of patients, if one does not perform the above mentioned calculation, true value of each component can not be obtained. When we want to know only the decrease or increase of some components from their apparent percentages, the correct results are obtained for albumin and $\gamma$-globulin only. Of course conditions of electrophoresis, namely ionic strength and pH of buffer solution and protein concentration, must be the same in one series of experiments. Further, if we want to know something about mobilities, the calculation above mentioned is necessary because the apparent and true mobilities are different from each other in both ascending and discending boundaries. But when we want to omit rather complicated calculations, relative mobilities are near to their correct values in ascending boundaries. Absolute mobilities are obtained from these relative mobilities and the absolute value of albumin mobility obtained from discending boundaries.

The author thanks Prof. K. Kodama, Prof. J. Koana and Dr. H. Hirai for their valuable kindness during these investigations.

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Fig. 1. *Ascending Boundaries*

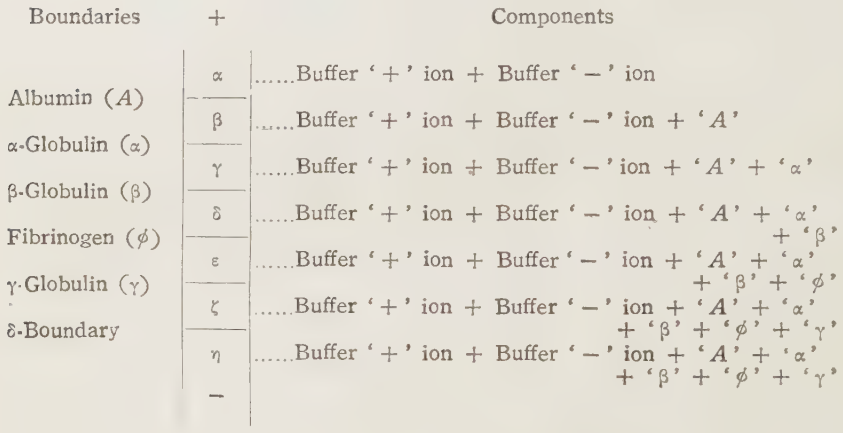


Table I. *$V_\sigma$ Products of Ascending Boundaries*

| Boundary | $V_\sigma$ products | | | |
|---|---|---|---|---|
| $A$ | $V^{\alpha\beta}{}_\sigma{}^\alpha = y_5$ | (1) | $V^{\alpha\beta}{}_\sigma{}^\beta = r_6 = r_{\text{alb.}}$ | (7) |
| α | $V^{\beta\gamma}{}_\sigma{}^\beta = y_4$ | (2) | $V^{\beta\gamma}{}_\sigma{}^\gamma = r_5 = r_{\alpha\text{-glob.}}$ | (8) |
| β | $V^{\gamma\delta}{}_\sigma{}^\gamma = y_3$ | (3) | $V^{\gamma\delta}{}_\sigma{}^\delta = r_4 = r_{\beta\text{-glob.}}$ | (9) |
| φ | $V^{\delta\varepsilon}{}_\sigma{}^\delta = y_2$ | (4) | $V^{\delta\varepsilon}{}_\sigma{}^\varepsilon = r_3 = r_{\text{fib.}}$ | (10) |
| γ | $V^{\varepsilon\zeta}{}_\sigma{}^\varepsilon = y_1$ | (5) | $V^{\varepsilon\zeta}{}_\sigma{}^\zeta = r_2 = r_{\gamma\text{-glob.}}$ | (11) |
| δ | $V^{\zeta\eta} = 0$ | (6) | $V^{\zeta\eta} = 0$ | (12) |

where $0 > y_1 > y_2 > y_3 > y_4 > y_5$ are roots of the equation

$$\frac{T_1{}^\eta}{r_1 - y} + \frac{T_2{}^\eta}{r_2 - y} + \frac{T_3{}^\eta}{r_3 - y} + \frac{T_4{}^\eta}{r_4 - y} + \frac{T_5{}^\eta}{r_5 - y} + \frac{T_6{}^\eta}{r_6 - y} + \frac{T_7{}^\eta}{r_7 - y} = 0 \quad \text{(I)}$$

TABLE II. *Concentration of each Component in each Phase of the Ascending Boundaries* ($C_i^{\nu}$)

| Component \ Phase | α | β | γ | δ | ε | ζ | η |
|---|---|---|---|---|---|---|---|
| Buffer ' + ' ion (1) | $C_1^{\beta}\frac{y_5(r_6-r_1)}{r_6(y_5-r_1)}$ | $C_1^{\gamma}\frac{y_4(r_5-r_1)}{r_5(y_4-r_1)}$ | $C_1^{\delta}\frac{y_3(r_4-r_1)}{r_4(y_3-r_1)}$ | $C_1^{\varepsilon}\frac{y_2(r_3-r_1)}{r_3(y_2-r_1)}$ | $C_1^{\zeta}\frac{y_1(r_2-r_1)}{r_2(y_1-r_1)}$ | $kC_1^{\eta}$ | $C_1^{\eta}$ |
| γ-Globulin (2) | 0 | 0 | 0 | 0 | 0 | $kC_2^{\eta}$ | $C_2^{\eta}$ |
| Fibrinogen (3) | 0 | 0 | 0 | 0 | $C_3^{\zeta}\frac{y_1(r_2-r_3)}{r_2(y_1-r_3)}$ | $kC_3^{\eta}$ | $C_3^{\eta}$ |
| β-Globulin (4) | 0 | 0 | 0 | $C_4^{\varepsilon}\frac{y_2(r_3-r_4)}{r_3(y_2-r_4)}$ | $C_4^{\zeta}\frac{y_1(r_2-r_4)}{r_2(y_1-r_4)}$ | $kC_4^{\eta}$ | $C_4^{\eta}$ |
| α-Globulin (5) | 0 | 0 | $C_5^{\delta}\frac{y_3(r_4-r_5)}{r_4(y_3-r_5)}$ | $C_5^{\varepsilon}\frac{y_2(r_3-r_5)}{r_3(y_2-r_5)}$ | $C_5^{\zeta}\frac{y_1(r_2-r_5)}{r_2(y_1-r_5)}$ | $kC_5^{\eta}$ | $C_5^{\eta}$ |
| Albumin (6) | 0 | $C_6^{\gamma}\frac{y_4(r_5-r_6)}{r_5(y_4-r_6)}$ | $C_6^{\delta}\frac{y_3(r_4-r_6)}{r_4(y_3-r_6)}$ | $C_6^{\varepsilon}\frac{y_2(r_3-r_6)}{r_3(y_2-r_6)}$ | $C_6^{\zeta}\frac{y_1(r_2-r_6)}{r_2(y_1-r_6)}$ | $kC_6^{\eta}$ | $C_6^{\eta}$ |
| Buffer ' − ' ion (7) | $C_i^{\beta}\frac{y_5(r_6-r_7)}{r_6(y_5-r_7)}$ | $C_i^{\gamma}\frac{y_4(r_5-r_7)}{r_5(y_4-r_7)}$ | $C_7^{\delta}\frac{y_3(r_4-r_7)}{r_4(y_3-r_7)}$ | $C_7^{\varepsilon}\frac{y_2(r_3-r_7)}{r_3(y_2-r_7)}$ | $C_i^{\zeta}\frac{y_1(r_2-r_7)}{r_2(y_1-r_7)}$ | $kC_7^{\eta}$ | $C_7^{\eta}$ |

Fig. 2. *Descending Boundaries*

| Boundary | + | Components |
|---|---|---|
| *A* | α | .....Buffer '+' ion + Buffer '−' ion + '*A*' + 'α' + 'β' + 'φ' + 'γ' |
| α | β | .....Buffer '+' ion + Buffer '−' ion + 'α' + 'β' + 'φ' + 'γ' |
| β | γ | .....Buffer '+' ion + Buffer '−' ion + 'β' + 'φ' + 'γ' |
| φ | δ | .....Buffer '+' ion + Buffer '−' ion + 'φ' + 'γ' |
| γ | ε | .....Buffer '+' ion + Buffer '−' ion + 'γ' |
| ε | ζ | .....Buffer '+' ion + Buffer '−' ion |
| | η | .....Buffer '+' ion + Buffer '−' ion |
| | − | |

TABLE III. *$V\sigma$ Products of Discending Boundaries*

| Boundary | $V'\sigma'$ Products | | | |
|---|---|---|---|---|
| *A* | $V^{\alpha\beta\prime}{}_{\sigma}{}^{\alpha\prime}=r_6$ | (13) | $V^{\alpha\beta\prime}{}_{\sigma}{}^{\beta\prime}=y_5$ | (19) |
| α | $V^{\beta\gamma\prime}{}_{\sigma}{}^{\beta\prime}=r_5$ | (14) | $V^{\beta\gamma\prime}{}_{\sigma}{}^{\gamma\prime}=y_4$ | (20) |
| β | $V^{\gamma\delta\prime}{}_{\sigma}{}^{\gamma\prime}=r_4$ | (15) | $V^{\gamma\delta}{}_{\sigma}{}^{\delta\prime}=y_3$ | (21) |
| φ | $V^{\delta\varepsilon\prime}{}_{\sigma}{}^{\delta\prime}=r_3$ | (16) | $V^{\delta\varepsilon\prime}{}_{\sigma}{}^{\varepsilon\prime}=y_2$ | (22) |
| γ | $V^{\varepsilon\zeta\prime}{}_{\sigma}{}^{\varepsilon\prime}=r_2$ | (17) | $V^{\varepsilon\zeta\prime}{}_{\sigma}{}^{\zeta\prime}=y_1$ | (23) |
| ε | $V^{\zeta\eta\prime}=0$ | (18) | $V^{\zeta\eta\prime}=0$ | (24) |

where $y_1, \ldots, y_5$ has the same value as in Table I

TABLE IV. *Cancentration of each Component in each Phase of the Discending Boundaries*

| Component \ Phase | α | β | γ | δ | ε | ζ | η |
|---|---|---|---|---|---|---|---|
| 1 | $C_1{}^{\alpha\prime}=C_1{}^{\eta}$ | $C_1{}^{\alpha\prime}\frac{y_5(r_6-r_1)}{r_6(y_5-r_1)}$ | $C_1{}^{\beta\prime}\frac{y_4(r_6-r_1)}{r_5(y_4-r_1)}$ | $C_1{}^{\gamma\prime}\frac{y_3(r_4-r_1)}{r_4(y_3-r_1)}$ | $C_1{}^{\delta\prime}\frac{y_2(r_3-r_1)}{r_3(y_2-r_1)}$ | $C_1{}^{\varepsilon\prime}\frac{y_1(r_2-r_1)}{r_2(y_1-r_1)}$ | $k'C_1\zeta'$ |
| 2 | $C_2{}^{\alpha\prime}=C_2{}^{\eta}$ | $C_2{}^{\alpha\prime}\frac{y_5(r_6-r_2)}{r_6(y_5-r_2)}$ | $C_2{}^{\beta\prime}\frac{y_4(r_5-r_2)}{r_5(y_4-r_2)}$ | $C_2{}^{\gamma\prime}\frac{y_3(r_4-r_2)}{r_4(y_3-r_2)}$ | $C_2{}^{\delta\prime}\frac{y_2(r_3-r_2)}{r_3(y_2\ r_2)}$ | 0 | 0 |
| 3 | $C_3{}^{\alpha\prime}C_3{}^{\eta}$ | $C_3{}^{\alpha\prime}\frac{y_5(r_6-r_3)}{r_6(y_5-r_3)}$ | $C_3{}^{\beta\prime}\frac{y_4(r_5-r_3)}{r_5(y_4-r_3)}$ | $C_3{}^{\gamma\prime}\frac{y_3(r_4-r_3)}{r_4(y_3-r_3)}$ | 0 | 0 | 0 |
| 4 | $C_4{}^{\alpha\prime}=C_4{}^{\eta}$ | $C_4{}^{\alpha\prime}\frac{y_5(r_6-r_4)}{r_6(y_5-r_4)}$ | $C_4{}^{\beta\prime}\frac{y_4(r_5-r_4)}{r_5(y_4-r_4)}$ | 0 | 0 | 0 | 0 |
| 5 | $C_5{}^{\alpha\prime}=C_5{}^{\eta}$ | $C_5{}^{\alpha\prime}\frac{y_5(r_6-r_5)}{r_6(y_5-r_5)}$ | 0 | 0 | 0 | 0 | 0 |
| 6 | $C_6{}^{\alpha\prime}=C_6{}^{\eta}$ | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | $C_7{}^{\alpha\prime}=C_7{}^{\eta}$ | $C_7{}^{\alpha\prime}\frac{y_5(r_6-r_7)}{r_6(y_5-r_7)}$ | $C_7{}^{\beta\prime}\frac{y_4(r_5-r_7)}{r_5(y_4-r_7)}$ | $C_7{}^{\gamma\prime}\frac{y_3(r_4-r_7)}{r_4(y_3-r_7)}$ | $C_7{}^{\delta\prime}\frac{y_2(r_3-r_7)}{r_3(y_2\ r_7)}$ | $C_7{}^{\varepsilon\prime}\frac{y_1(r_2-r_7)}{r_2(y_2-r_7)}$ | $k'C_7\zeta'$ |

TABLE V.

| Sample | Protein Content-ration gr./dl. | Relative* Resistance | | Boundary displacement* | | | | | $-V\sigma$ * | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | $A_a$ | $\alpha_a$ | $\beta_a$ | $\phi_a$ | $\gamma_a$ | $y_5$ | $y_4$ | $y_3$ | $y_2$ | $y_1$ |
| | | Sample | Buffer | $A_d$ | $\alpha_d$ | $\beta_d$ | $\phi_d$ | $\gamma_d$ | $r_6$ | $r_5$ | $r_4$ | $r_3$ | $r_2$ |
| Normal A | 3.3 | 1680 | 1600 | 17.0 | 13.3 | 9.6 | 7.4 | 3.9 | 1062 | 740 | 531 | 405 | 211 |
| | | | | 15.9 | 11.0 | 7.8 | 5.9 | $3.0_3$ | 947 | 735 | 526 | 402 | 208 |
| Normal A | 2.5 | 1630 | 1600 | 14.5 | 11.4 | 8.1 | 6.0 | 3.1 | 906 | 685 | 486 | 357 | 184 |
| | | | | 14.2 | 10.7 | $7.5_2$ | $5.5_0$ | 2.8 | 872 | 683 | 482 | 356 | 182 |
| Normal B | 3.2 | 1730 | 1605 | 14.0 | 11.3 | 8.0 | 5.9 | 3.0 | 869 | 603 | 425 | 310 | 158 |
| | | | | 12.9 | 8.9 | 6.2 | $4.5_3$ | $2.2_8$ | 748 | 600 | 420 | 310 | 156 |
| Normal B | 2.3 | 1660 | 1600 | 13.3 | 10.6 | 7.8 | 5.6 | 2.9 | 831 | 586 | 432 | 308 | 160 |
| | | | | 12.2 | 8.6 | 6.3 | 4.5 | 2.3 | 735 | 586 | 429 | 308 | 157 |
| Normal C | 3.4 | 1830 | 1720 | 15.4 | 12.3 | 8.9 | 6.6 | 3.5 | 895 | 628 | 450 | 326 | 171 |
| | | | | 14.4 | 10.0 | 7.0 | 5.0 | 2.6 | 787 | 622 | 440 | 322 | 170 |
| Inanition | 2.1 | 1560 | 1510 | 15.4 | 12.2 | 8.9 | 7.0 | 3.5 | 1020 | 758 | 552 | 428 | 213 |
| | | | | 14.9 | 11.0 | 7.9 | 6.1 | $3.0_1$ | 955 | 756 | 545 | 427 | 211 |
| Hyper-nephrom | 2.7 | 1885 | 1799 | 15.7 | 13.3 | 8.9 | 6.8 | 3.6 | 874 | 672 | 445 | 336 | 178 |
| | | | | 15.0 | 11.4 | $7.4_5$ | 5.6 | $2.9_2$ | 795 | 633 | 442 | 335 | 176 |
| Normal D | 2.0 | 1645 | 1600 | 17.0 | 13.2 | 9.7 | 6.9 | 3.6 | 1700 | 1190 | 872 | 616 | 321 |
| | | | | 15.8 | 11.0 | 8.0 | $5.6_4$ | $2.9_2$ | 1532 | 1185 | 865 | 614 | 319 |
| Normal D | 3.4 | 1710 | 1600 | 16.0 | 12.4 | 9.3 | 6.6 | 3.5 | 1600 | 1080 | 806 | 568 | 300 |
| | | | | 14.9 | 10.4 | 7.4 | 5.2 | $2.7_4$ | 1395 | 1073 | 800 | 566 | 295 |

* Arbitrary unit.

** $A_l$, $\alpha_l$, etc.; true relative mobility of albumin, $\alpha$-globulin, etc.

TABLE V (Continued)

| Apparent % | | | | | True % | | | | | —Relative mobility** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $A_a$ | $\alpha_a$ | $\beta_a$ | $\phi_a$ | $\gamma_a$ | $A_a$ | $\alpha_a$ | $\beta_a$ | $\phi_a$ | $\gamma_a$ | $A_t$ | $\alpha_t$ | $\beta_t$ | $\phi_t$ | $\gamma_t$ |
| $A_d$ | $\alpha_d$ | $\beta_a$ | $\phi_d$ | $\gamma_d$ | $A_d$ | $\alpha_a$ | $\beta_d$ | $\phi_d$ | $\gamma_d$ | $A_a$ / $A_d$ | $\alpha_a$ / $\alpha_a$ | $\beta_a$ / $\beta_d$ | $\phi_t$ / $\phi_d$ | $\gamma_a$ / $\gamma_d$ |
| 58.4 | 8.4 | 10.1 | 6.4 | 17.2 | 53.2 | 9.8 | 10.9 | 7.8 | 19.0 | 100 | 78 | 56 | 43 | 22 |
| 57.5 | 8.7 | 11.4 | 6.8 | 18.6 | 52.3 | 11.1 | 12.3 | 8.9 | 19.4 | 100 | 78 | 56 | 44 | 23 |
| 56.2 | 8.7 | 11.4 | 6.8 | 16.8 | 53.0 | 9.2 | 12.4 | 7.2 | 17.8 | 100 | 67 | 49 | 37 | 19 |
| 54.5 | 8.7 | 11.4 | 6.8 | 18.5 | 52.7 | 9.6 | 11.8 | 7.0 | 18.9 | 100 | 78 | 55 | 41 | 21 |
| 55.5 | 8.5 | 10.3 | 5.2 | 20.5 | 51.5 | 9.4 | 12.0 | 5.1 | 22.1 | 100 | 79 | 56 | 41 | 21 |
| 58.2 | 7.3 | 11.7 | 3.5 | 19.3 | 51.9 | 10.4 | 13.3 | 4.2 | 20.1 | 100 | 75 | 53 | 38 | 20 |
| 53.6 | 9.6 | 10.0 | 4.6 | 22.2 | 51.2 | 9.2 | 11.0 | 4.4 | 23.4 | 100 | 80 | 56 | 41 | 21 |
| 57.2 | 7.2 | 10.4 | 4.3 | 20.7 | 51.4 | 10.4 | 11.8 | 4.7 | 21.5 | 100 | 81 | 57 | 42 | 22 |
| 50.4 | 7.8 | 13.0 | 9.5 | 19.6 | 41.0 | 9.2 | 15.5 | 11.3 | 20.4 | 100 | 69 | 48 | 35 | 18 |
| 51.8 | 7.6 | 15.0 | 8.5 | 17.4 | 42.7 | 10.2 | 16.8 | 9.6 | 20.0 | 100 | 80 | 58 | 42 | 22 |
| 49 | 9 | 16 | 8 | 19 | 46 | 10 | 17 | 8 | 18 | 100 | 80 | 59 | 42 | 22 |
| 52 | 9 | 16 | 6 | 17 | 48 | 11 | 17 | 7 | 18 | 100 | 71 | 52 | 37 | 19 |
| 38.7 | 12.7 | 14.0 | 12.9 | 21.7 | 34.0 | 14.6 | 15.0 | 13.2 | 23.4 | 100 | 79 | 56 | 41 | 22 |
| 40.0 | 12.6 | 14.4 | 12.3 | 20.7 | 32.0 | 16.8 | 15.8 | 13.8 | 21.5 | 100 | 80 | 58 | 43 | 23 |
| 51.8 | 9.2 | 12.8 | 7.7 | 18.5 | 48.8 | 9.7 | 13.8 | 8.3 | 19.3 | 100 | 69 | 49 | 35 | 18 |
| 54.2 | 7.0 | 13.2 | 7.1 | 18.6 | 47.7 | 10.4 | 14.9 | 7.8 | 19.4 | 100 | 79 | 57 | 45 | 22 |
| 53.5 | 8.6 | 12.4 | 6.7 | 18.8 | 49.5 | 9.6 | 13.0 | 7.3 | 20.7 | 100 | 79 | 58 | 45 | 23 |
| 56.6 | 7.3 | 11.6 | 6.1 | 18.4 | 49.8 | 10.4 | 13.8 | 6.9 | 19.2 | 100 | 74 | 53 | 41 | 20 |
| | | | | | | | | | | 100 | 83 | 56 | 42 | 22 |
| | | | | | | | | | | 100 | 85 | 57 | 43 | 23 |
| | | | | | | | | | | 100 | 76 | 50 | 37 | 19 |
| | | | | | | | | | | 100 | 78 | 56 | 40 | 21 |
| | | | | | | | | | | 100 | 78 | 57 | 41 | 21 |
| | | | | | | | | | | 100 | 70 | 51 | 36 | 18 |
| | | | | | | | | | | 100 | 77 | 57 | 41 | 21 |
| | | | | | | | | | | 100 | 78 | 58 | 41 | 22 |
| | | | | | | | | | | 100 | 70 | 50 | 35 | 18 |

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# A NEW METHOD FOR THE COLORIMETRIC DETERMINATION OF ARGININE.

By

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About 20 years ago, I reported a colour reaction given by arginine on the addition of $\alpha$-naphtol and sodium hypochlorite to its alkaline solution (1). This reaction was developed by Weber (2) to the colorimetric determination of arginine with the modification of using hypobromite instead of hypochlorite. This method has, however, a disadvantage in that $\alpha$-naphtol gradually produces a yellow colour on the addition of hypobromite, which interferes the arginine determination. In this communication I propose the use of oxine instead of $\alpha$-naphtol, as oxine remains colourless on the mere addition of hypobromite.

### *The new colour reaction of arginine.*

If oxine (8-oxychinolin) and sodium hypochlorite or hypobromite are added to an alkaline solution of arginine, a reddish brown colour results. This colour reaction has no advantage over the former with regards to the sensibility, which remains almost the same in both reactions (1: 2,500,000). As the qualitative test the new reaction is rather inferior to the former owing to its yellow coloration in the case of the dilute arginine solution. The reaction has, however, a distinct advantage that the developed colour is stable enough to allow the exact colorimetric determination for certain length of time.

The procedures of the new method is principally the same as Webers method. For the colour comparison the Pulfurichs photometer was used with the filter S 50 against water. The relation

between concentration and extinction coefficient was studied on solutions containing 1–10 γ of arginine per cc. The results of these observations are given in Table I, where the values under A and B rowe were determined under different condition, which will be explained later.

TABLE I.

| Amount of arginine in 5 ml. | Extinction coefficient |
|---|---|
| 5 γ | 0,10 |
| 10 | 0,21 |
| 20 | 0,45 |
| 30 | 0,65 |
| 40 | 0,85 |
| 50 | 1,09 |

As the Table I shows the extinction coefficient in both conditions in quite proportional to the amount of arginine following the Lambert-Beer's law, and any arginine solution of unknown concentration can be determined from its extinction coefficient, if once an extinction coefficient curve is worked out with arginine solution of known concentrations.

The hydrolysate of protein contains many substances preventing the full developement of the colour, among which ammonia is most remarkable. Experiments were therefore carried out first to investigate the effect of ammonia on the developement of colour. The amount of ammonia which was added in the form of ammonium sulfate varied 0,05–0,3 mg. in 5 ml. of arginine solution (6 γ/ml.).

As shown in Table II, the interference of ammonia of high dilution is comparatively small with the auther's method, while this is not the case with other methods.

TABLE II.

*The effect of the presence of ammonia on the different colorimetric methods.*

| Colorimetric method of arginine | Ammonia-N (mg.) in 5 ml. of arginine solution (6 γ/ml.) | | | |
|---|---|---|---|---|
| | 0,05 | 0,1 | 0,2 | 0,3 |
| | % | % | % | % |
| The author's method | 99 | 92 | 73 | 57 |
| The method of Fisher and Wilhelmi (3) | 76 | 67 | 53 | 32 |
| The method of Thomas, Ingalls and Luck (4) | 75 | 65 | 50 | 31 |

TABLE III.

*The arginine contents of several proteins obtained by the new colorimetric method and its comparison with those by Vickery's gravimetric method (5).*

| | The new method | The Vickery's method |
|---|---|---|
| | % | % |
| Egg Albumin | 5,26 | 5,20 |
| Edestin | 15,81 | 16,05 |
| Fibrin | 6,15 | 6,38 |
| Gliadin | 2,25 | 2,37 |
| Serum Globulin | 3,85 | 4,10 |
| Gelatin | 8,10 | 8,25 |
| Hemoglobin | 3,33 | 3,25 |
| Wool | 10,15 | 10,45 |
| Caseinogen | 3,45 | 3,25 |

Next, the effect of other substances contained in the protein hydrolysate was tested by the comparison of the values of the arginine contents determined both by the new colorimetric method and by the Vickery's gravimetric method (Table III.)

Date indicate that they agree fairly well each other. It may be postulated, therefore, that this colorimetric method can be applied for the hydrolysates of protein with good result.

*The method of determination of arginine.*

Solution required: (1) Sodium hydroxide, 10 per cent; (2) oxine, 0,02 per cent, prepared by diluting 0,4 per cent alcoholic solution of oxine with water; (3) sodium hypobromite, prepared by dissolving 2 g. of bromine in 100 ml. of 5 per cent sodium hydroxide solution; (4) urea, 40 per cent. (5) standard solution, 24, 2 mg. arginine hydrochloride $C_6H_{14}O_2N_4 \cdot HCl$ are transferred to the measuring flask of 200 ml. capacity, added with 10 ml. of 10 per cent hydrochloric acid and made up to the volume with distilled water (stock solution). This contains 100 $\gamma$ arginine per ml. and keeps for several months. For use this standard solution is diluted each time freshly to the concentration required.

Procedure: Method A.—5 ml. of the solution to be tested are pipetted into a testtube (2×18 cm.) placed in an ice bath. After the addition of 1 ml. of oxine solution and 1 cc. of sodium hydroxide solution the contents of the tube are cooled in the ice bath for 2 or 3 min. Then a proper amount of hypobromite is added, the tube shaken, and 20 sec. later 1 ml. urea is added and thoroughly mixed. Because the presence of urea does not completely prevent fading the colorimetric comparison should be carried out within 5 min. The result is given in Table I., A., where pure arginine solution was used.

But this method offers some trouble in reading the extinction coefficient in the wet season as met in summer of Japan owing to

the condensation of dew on the surface of cubett containing the fluid under examination. In this case the following modification is rather recommended.

Method B—5 ml. water are added exact 1 min. after the addition of urea with shaking. The developed maximum colour lasts for a very considerable time. This method offers no trouble even in summer season.

A suitable concentration of arginine is in a range 1–10 $\gamma$ per ml., so that the dilution of protein hydrolysates of 1: 20,000–40,000 is found satisfactory. The proper amount of hypobromite must be worked out in each unknown solution. This is done by taking a series of tubes containing 5 ml. of the solution to be tested and increasing the amount of hypobromite drop by drop. This amount is usually 2 drops for the standard solution and 2–4 drops for the protein hydrolysates.

This colorimetric method of arginine can be also applicable to the determination of glycocyamin.

*Hydrolysis of protein.*

When the colorimetric determination of arginine in protein is attempted, the protein must be first hydrolysed by acid. If 20 per cent hydrochloric acid is used for this purpose, the complete hydrolysis is usually attained by boiling over twenty hours under reflux condenser. In the course of my investigation, however, I found that there is no difference on the arginine reaction between partial and total hydrolysates of protein, the partial hydrolysis being performed by heating with 10 per cent hydrochloric acid three hours at 100°. By this observation protein hydrolysis is carried out as follows: 1–100 mg. samples are weighed out into a small testtube (1×10 cm.), (Fig. 1) added with 3 ml. of 20 per cent hydrochloric acid and heated for four hours on a waterbath. After the lapse of 15–20 min., contents are carefully stirred with a glass rod, then a glass cap


Fig. 1.

is putted on the tube and the heating is continued being the tube occassionally shaken to give a complete mixture of the content. The products of hydrolysis are filtered and the insoluble residue is thoroughly washed with hot water. The combined filtrates are roughly neutralized by cautious addition of dilute caustic soda and diluted to the suitable concentration as stated above and using 5 ml. of this solution the determination is to be carried out.

To apply this method to the determination of arginine in tissue protein a peace of tissue is weighed out into a centrifuge tube, treated with alcohol and ether to get free of fat and dried. The residue in the centrifuge tube is submitted to hydrolysis as stated above and after suitable dilution the determination is carried out. It is also advisable to check the accuracy of the method by the recovery test.

## Summary.

1. A new colorimetric method for the determination of arginine based on a colour reaction with oxine and sodium hypobromite is reported.

2. A simple method of hydrolysis of protein for the arginine determination is reported.

In conclusion I wish to express my hearty thanks to Emer. prof. S. Kakiuchi and Prof. K. Kodama for their kind advice.

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# ON THE PROTEASE ACTION OF PENICILLIUM NOTATUM. II.

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As regards the peptidase activity of molds, investigations were done by Abderhalden *et al.* (1), Schmalfuss *et al.* (2) and Otani (3) into Asp. niger while Yamaguchi (4) and Wada (5) researched into Pen. glaucum. On the other hand, the existence of hippuricase was proved in various fields by various scholars, namely, in Asp. niger by Shibata (6), in various species of Penicillia as well as in Asp. by niger Dox (7), in Pen. glaucum by Yamaguchi (4) and in Asp. oryzae by Neuberg *et al.* (8). Otani (9) has found the splitting-ability of various species of molds on the benzoyl-derivatives of various peptides. The detailed studies followed by Johnson *et al.* (10, 11) and Berger et al. (12) of the proteolytic enzymes of many common molds have led them to conclude that the proteolytic system of Asp. parasiticus consists of at least one proteinase and five peptidases; namely, a dipeptidase, a carboxy-polypeptidase, an aminopolypeptidase and two enzymes that hydrolyze diglycine and triglycine. They studied the specificities of its purified aminopolypeptidase only to find out that Asp. parasiticus hydrolyses neither benzoyldiglycine nor benzoyltriglycine. The purified preparation, made by acetone precipitation from the 4 month autolyzate of mycelium of Penicillium strains No. 50, No. 233 and NRRL $B_2$, as Tazawa *et al.* (13) reported, attacked gelatin remarkably, but never glycyl-DL-asparagin, L-leucyldiglycine, benzoyl-glycine and benzoyldiglycine at all.

Having described on the proteinase and the peptonase action of Pen. notatum in the previous article (14), the author would like to report in this present paper the results obtained from his continuous research into the peptidase actions of Pen. notatum.

## Experimental.

### Table I.

Protease action of Pen. notatum (Q 176).

Enzyme: Pen. notatum, P 176 and Q 176, cultivated as described in the preivous report, were used as enzyme sources. The mycelium, which was washed repeatedly with physiological NaCl solution, was ground in a mortar with 2 times mycelium volume of glycerine-water (1:1) and was filtered through gauze. The maceration thus obtained was stored under toluene in the ice box and used in the experiments.

Test solutions: 5 ml. 4% protein or 0.2 *M* peptide solution+5 ml. maceration+10 ml. buffer solution (pH 6.0 and 7.5)+toluene; incubated at 37°. Control solution, containing only enzyme and buffer without substrate, was treated in just the same way.

After a definite time, the acidity increase in 4 ml. digestate was measured by the formol titration with 0.1 *N* NaOH solution. The values obtained from the control experiments were deducted from those of the main experiments, and those corrected values of the acidity increase are shown in the following table.

Benzoic acid was isolated from the digestate in the following way. 100 ml. of 0.05 *M* hippuric acid solution (as Na-salt) was mixed with 10 ml. of maceration prepared with 3 times volume of glycerine-water (1:1) and the mixture (pH 7.5) was incubated at 37° under toluene after adjusting to pH 7.5 for 5 days. Then, the test mixture was again digested after addition of 10 ml. of maceration. After the second incubation, which showed no more acidity increase, the mixture was filtered and the filtrate was extracted with ether at HCl acid reaction. 0.18 g. of crystalline substance was obtained after evaporation of ether. M. p. 121° after recrystallization from hot water; no depression with benzoic acid.

Acidity increase in 4 ml. digestate (ml. 0.1 *N* NaOH)

| Substrate \ pH / Time hrs. | 6.0 | | | 7.5 | | |
|---|---|---|---|---|---|---|
| | 24 | 72 | 120 | 24 | 72 | 120 |
| Gelatin | 0.44 | 0.82 | 0.87 | 0.45 | 0.80 | 0.88 |
| Peptone (Riedel) | 1.06 | 1.50 | 1.62 | 1.02 | 1.27 | 1.29 |
| Peptone T. | 0.14 | 0.26 | 0.39 | 0.13 | 0.18 | 0.20 |
| Protamine | 0.41 | 1.04 | 1.41 | 0.42 | 0.72 | 0.84 |
| Glycylglycine | 0.14 | 0.23 | 0.31 | 0.58 | 0.76 | 0.95 |
| Triglycine | 1.30 | 1.60 | 1.76 | 1.61 | 1.93 | 2.21 |
| Leucyldiglycine | 0.46 | 0.46 | 0.55 | 0.39 | 0.42 | 0.59 |
| Benzoylglycine | 0.25 | 0.85 | 1.25 | 0.37 | 1.10 | 1.59 |
| Benzoyl-DL-methionine* | 0 | 0.06 | 0.10 | 0 | 0 | 0 |
| Benzoyl-DL-phenylalanine* | 0 | 0 | 0.09 | 0 | 0 | 0 |
| Benzoyldiglycine | 0.07 | 0.31 | 0.55 | 0.09 | 0.16 | 0.16 |
| Acetylglycine | 0.28 | 0.41 | 0.68 | 0.17 | 0.29 | 0.43 |
| Acetylglutamic acid | 0.50 | 0.52 | 0.67 | 0.17 | 0.17 | 0.17 |
| Acetyl-DL-methionine* | 0 | 0 | 0 | 0 | 0 | 0 |
| Cl-acetylleucine | 0.95 | 0.98 | 1.13 | 0.62 | 0.68 | 0.80 |
| Cl-acetylphenylalanine | 0.22 | 0.66 | 0.78 | 0.71 | 0.83 | 0.93 |

* Pen. notatum, Q 176 N-3 strain, was used.

TABLE II.

Peptidase action of maceration and acetone powder (P 176 and Q 176).

Enzyme: Maceration prepared with 3 times mycelium volume of glycerine-water (1 : 1).

The well-ground mycelium was treated with 3 times its volume of acetone with stirring and centrifuged down. The operation was repeated three times, and then it was finally treated with ether. It was filtered by suction and dried over $H_2SO_4$ in a vacuum desiccator after evapora-

tion of ether. The acetone powder was used as 2 % water suspension in the experiments.

Test solutions: 5 ml. 0.2 *M* peptide solution+2 ml. maceration or 2 ml. 2% water suspension of acetone powder+13 ml. buffer solution of vorious pH values; incubated at 37°.

Acidity increase in 4 ml. digestate (ml. 0.1 *N* NaOH)

| Substrate | Enzyme | pH / Time hrs. | 4.0 | 5.0 | 6.0 | 6.5 | 7.0 | 7.5 | 7.8 | 8.0 | 9.0 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Diglycine | Acetone powder (P 176) | 24 | — | — | 0.04 | — | — | 0.09 | — | — | — |
| | | 72 | — | — | 0.14 | — | — | 0.15 | — | — | — |
| | | 120 | — | — | 0.17 | — | — | 0.26 | — | — | — |
| | Maceration (P 176) | 24 | 0 | 0 | 0.08 | — | 0.10 | 0.17 | 0.25 | 0.18 | 0 |
| | | 72 | 0 | 0 | 0.18 | — | 0.23 | 0.53 | 0.45 | 0.21 | 0 |
| | | 120 | 0 | 0 | 0.20 | — | 0.26 | 0.69 | 0.47 | 0.23 | 0 |
| Benzoyl-glycine | Acetone powder (P 176) | 24 | — | — | 0.57 | — | — | 0.41 | — | — | — |
| | | 72 | — | — | 1.36 | — | — | 1.09 | — | — | — |
| | | 120 | — | — | 1.69 | — | — | 1.58 | — | — | — |
| | Maceration (P 176) | 24 | 0 | 0.15 | 0.20 | 0.28 | 0.30 | 0.29 | — | 0.08 | 0 |
| | | 72 | 0 | 0.17 | 0.62 | 0.63 | 0.86 | 0.83 | — | 0.10 | 0 |
| | | 120 | 0 | 0.28 | 0.77 | 0.73 | 1.34 | 1.12 | — | 0.12 | 0 |
| Benzoyl-diglycine | Acetone powder and Maceration (P 176) | 24 | — | 0 | 0 | 0 | 0 | 0 | — | 0 | 0 |
| | | 72 | — | 0 | 0 | 0 | 0 | 0 | — | 0 | 0 |
| | | 120 | — | 0 | 0 | 0 | 0 | 0 | — | 0 | 0 |
| | Maceration* (Q 176) | 24 | 0 | 0.05 | 0.13 | — | 0.10 | — | — | — | — |
| | | 72 | 0 | 0.05 | 0.28 | — | 0.24 | — | — | — | — |
| | | 120 | 0 | 0.10 | 0.39 | — | 0.33 | — | — | — | — |

* In this experiment, 5 ml. of maceration prepared with 2 times volume of glycerine-water (1:1) and 10 ml. of buffer solution were used.

TABLE III.

Proteolytic action (Q 176) of acid treated maceration (pH 4.0).

Enzyme: Maceration was prepared with 2 times mycelium volume of distilled water.

After adjusting to pH 4.0 with 3% HCl, the enzyme solution was incubated at 37° for a definite time, it was then put back to the original pH with 3 % NaOH and used.

Test solutions: 5 ml. 4% protein or 0.2 *M* peptide solution+5 ml. maceration+10 ml. buffer solution (pH 6.0 and 7.5)+toluene; incubated at 37°.

Acidity increase in 4 ml. digestate (ml. 0.1 *N* NaOH)

| Time of acid treatment min. | Substrate \ pH / Time hrs. | 6.0 | | | 7.5 | | |
|---|---|---|---|---|---|---|---|
| | | 24 | 72 | 120 | 24 | 72 | 120 |
| 5 | Gelatin | 0 | 0 | 0 | 0 | 0 | 0 |
| | Peptone (Riedel) | 0.22 | 0.63 | 0.64 | 0.10 | 0.31 | 0.35 |
| | Peptone (T.) | 0 | 0 | 0 | 0 | 0 | 0 |
| | Glycylglycine | 0 | 0 | 0 | 0 | 0 | 0 |
| | Benzoylglycine | 0 | 0.33 | 0.41 | 0 | 0.23 | 0.27 |
| | Benzoyldiglycine | 0 | 0 | 0 | 0 | 0 | 0 |
| 60 | Gelatin | 0 | 0 | 0 | 0 | 0 | 0 |
| | Peptone (Riedel) | 0.08 | 0.35 | 0.55 | 0.05 | 0.24 | 0.31 |
| | Peptone (T.) | 0 | 0 | 0 | 0 | 0 | 0 |
| | Glycylglycine | 0 | 0 | 0 | 0 | 0 | 0 |
| | Benzoylglycine | 0 | 0.10 | 0.40 | 0.02 | 0.11 | 0.40 |
| | Benzoyldiglycine | 0 | 0 | 0 | 0 | 0 | 0 |
| 120 | Gelatin | 0 | 0 | 0 | 0 | 0 | 0 |
| | Peptone (Riedel) | 0 | 0.16 | 0.33 | 0 | 0 | 0.13 |
| | Peptone (T.) | 0 | 0 | 0 | 0 | 0 | 0 |
| | Glycylglycine | 0 | 0 | 0 | 0 | 0 | 0 |
| | Benzoylglycine | 0 | 0.10 | 0.16 | 0 | 0.05 | 0.16 |
| | Benzoyldiglycine | 0 | 0 | 0 | 0 | 0 | 0 |

TABLE IV.

Proteolytic action of alkaline treated maceration (pH 9.5 and 11.5).

Enzyme: Maceration was preliminary incubated at pH 9.5 or at 11.5 (NaOH) for a certain time and neutralized, then tested in the experiment.

Test solutions: The same as described in Table III.

Acidity increase in 4 ml. digestate (ml. 0.1 *N* NaOH)

| pH and time of treatment | Substrate \ pH / Time hrs. | 6.0 | | | 7.5 | | |
|---|---|---|---|---|---|---|---|
| | | 24 | 72 | 120 | 24 | 72 | 120 |
| pH 9.5 1 hr. | Gelatin | 0.30 | 0.51 | 0.77 | 0.35 | 0.50 | 0.60 |
| | Peptone (Riedel) | 0.99 | 1.17 | 1.21 | 0.81 | 0.86 | 0.86 |
| | Peptone (T.) | 0.18 | 0.22 | 0.22 | 0.09 | 0.09 | 0.09 |
| | Glycylglycine | 0 | 0.21 | 0.37 | 0.17 | 0.55 | 0.97 |
| | Benzoylglycine | 0 | 0.21 | 0.54 | 0 | 0 | 0.35 |
| | Benzoyldiglycine | 0.03 | 0.21 | 0.48 | 0 | 0 | 0 |
| pH 9.5 17 hrs. | Gelatin | 0.27 | 0.49 | 0.72 | 0.05 | 0.27 | 0.36 |
| | Peptone (Riedel) | 0.99 | 1.08 | 1.16 | 0.87 | 0.88 | 1.01 |
| | Peptone (T.) | 0.12 | 0.15 | 0.34 | 0 | 0 | 0 |
| | Glycylglycine | 0.15 | 0.42 | 0.48 | 0.67 | 0.82 | 1.00 |
| | Benzoylglycine | 0 | 0.14 | 0.33 | 0 | 0.19 | 0.39 |
| | Benzoyldiglycine | 0.03 | 0.17 | 0.32 | 0 | 0 | 0 |
| pH 11.5 1 hr. | Gelatin | 0 | 0 | 0 | 0 | 0 | 0 |
| | Peptone (Riedel) | 0.08 | 0.33 | 0.45 | 0.17 | 0.33 | 0.41 |
| | Peptone (T.) | 0 | 0 | 0 | 0 | 0 | 0 |
| | Glycylglycine | 0 | 0 | 0 | 0 | 0.40 | 0.43 |
| | Benzoylglycine | 0 | 0 | 0.27 | 0 | 0.07 | 0.20 |
| | Benzoyldiglycine | 0 | 0 | 0 | 0 | 0 | 0 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| pH 11.5 2 hrs. | Gelatin | 0 | 0 | 0 | 0 | 0 | 0 |
| | Peptone (Riedel) | 0.18 | 0.40 | 0.50 | 0.34 | 0.47 | 0.56 |
| | Peptone (T.) | 0 | 0 | 0 | 0 | 0 | 0 |
| | Glycylglycine | 0 | 0 | 0 | 0 | 0 | 0 |
| | Benzoylglycine | 0 | 0 | 0.15 | 0 | 0.11 | 0.23 |
| | Benzoyldiglycine | 0 | 0 | 0 | 0 | 0 | 0 |

## Results and Considerations.

The observations were specially concerned with peptidase action of the maceration of notatum mycelium (P 176 and Q 176) as well as the acetone powder prepared by means of acetone-ether method. And examination was also made of the resistance of the enzyme against acid or alkaline treatment.

The results as indicated in Table I, were obtained in the study of the proteolytic actions of notatum (Q 176) maceration at pH 6.0 and 7.5. As proteins, protamine was hydrolyzed remarkably besides gelatin and peptone. Among glycine-peptides, both diglycine and triglycine were hydrolyzed easily, especially at alkaline side and leucyldiglycine at both sides though weakly. Benzoylglycine was markedly hydrolyzed. Benzoyldiglycine was attacked at pH 6.0 though not so remarkably, but was hardly attacked at pH 7.5. Acetylglycine and acetylglutamic acid were split, but the latter showed only slight acidity increase at alkaline side. Cl-acetylleucine and Cl-acetylphenylalanine were also broken down. From those observations, the existence of hippurase, acylase and carboxypeptidase, which had been proved in animal tissues, was acknowledged also in Penicillium. It must be noted that benzoyl-DL-methionine, benzoyl-DL-phenylalanine and acetyl-DL-methionine were not attacked by the Penicillium enzyme (Q 176 N-3 strain).

As Table II shows, the optimum pH for the hydrolysis of glycylglycine was found to be at pH 7.5, that for benzoylglycine at

pH 7.0 and that for benzoyldiglycine (Q 176) near at pH 6.0. Hippuric acid was very easily attacked by the maceration and also by the acetone powder, which seemed to undergo partial inactivation and could scarcely break down glycylglycine. It must be noted here that Penicillium shows a more remarkable activity of hippurase than that of dipeptidase.

Benzoyldiglycine was not split at both sides by P 176 strain, but was only weakly hydrolyzed at acid side by Q 176. Acetone powder had no ability. It should also be remarked here that the ability of benzoyldiglycine break down (perhaps carboxypeptidase) was found in a strain of the molds.

According to the report of Otani (9), with the rarest exception, benzoylglycine is hydrolyzed far more easily by molds than benzoyldiglycine. The above mentioned results of Johnson et al. (11) also indicate that Asp. parasiticus cannot attack benzoyldiglycine. The results obtained here have perfectly coincided with these observations. Even if benzoyldiglycine is hydrolyzed by a certain strain, the grade is very weak in comparison with hippurase activity as table shows. The investigations by Utzino *et al.* (15, 16) show that the pancreatic enzymes can split benzoyldiglycine but not benzoylglycine, and that the carboxypeptidase, therefore, must be distinguished from hippurase. These observations indicate that the appearance of these both enzymes is different in animals from that in molds.

It is reported by Kimura (17) that acetylamino acids are far more easily hydrolyzed by animal organs than benzoylglycine. On the other hand, Imaizumi (18) found various cases about Bac. prodigiosus, Bac. coli, Bac. proteus and Staphylococcus aureus and the author (19) also found that Bac. Natto generally could easily attack benzoyl-derivatives but hardly attacked acetylamino acids.

Then, here the resistance of enzymes against acid and alkali has been investigated, in order to know the properties of the enzymes and further to distinguish the enzyme specificity. It is reported by

Johnson (10) that the proteinase of Asp. parasiticus is neither alkali- nor acidstable and that when proteinase was kept at room temperature 14 hrs. long, it lost at pH 3.5, 95% and at pH 9.5, 80% in activity. The proteinase of green mold, Tazawa *et al.* (13) observed, is stable for acid and alkali between pH 5.0 and 8.0, but decreases gradually its activity according to the increase in acidity or alkalinity. In the present work, gelatin, peptone (Riedel and Teruuchi), glycylglycine, benzoylglycine and benzoyldiglycine were tested as substrates. In case of acid treatment (Table III), only two activities of peptone (Riedel) and benzoylglycine-splitting at pH 6.0 and 7.5 remained and the others, dipeptidase, hippurase and carboxypeptidase, disappeared, when the maceration was kept at pH 4.0 and 37° for 5 to 60 minutes. Even those two actions were damaged by incubation at pH 4.0 and 37° for 90 to 120 minutes. These observations denote that the mold enzymes are very unstable against acid.

In case of alkaline treatment (Table IV), the enzymes seem to be resistant to alkaline incubation at pH 9.5 and 37° for 1 to 17 hrs. When the maceration, was incubated at pH 11.5 and 37°, it diminished considerably each splitting-ability after incubation for 30 minutes. It was not able to attack gelatin and benzoyldiglycine, but slightly glycylglycine at alkaline side after incubation for 60 minutes. After treatment for 2 hours it left only activity of peptone (Riedel)- and benzoylglycine-splitting. On the whole, peptone (Riedel)- as well as benzoylglycine-splitting activity have resistance against acid and alkali.

## Summary.

1) The maceration of Pen. notatum hydrolyzes protamine besides gelatin and peptone as proteins. It contains also dipeptidase (glycylglycine), tripeptidase (triglycine, leucyldiglycine), hippurase, carboxydipeptidase, acylase (acetylglycine, acetylglutamic acid) and halogenacylase (Cl-acetylleucine, Cl-acetylphenylalanine).

2) Both maceration and acetone powder are able to attack diglycine and benzoylglycine. The hippurase has rather resistance against acetone treatment contrary to the dipeptidase. The optimum pH for the hydrolysis of glycylglycine is found to be at pH 7.5, that for benzoylglycine at pH 7.0 and that for benzoyldiglycine near at pH 6.0. Benzoic acid was isolated as the product of hippurase action.

3) Moldenzymes are unstable against acid, leaving only peptone (Riedel)- and benzoylglycine-splitting activity by keeping at pH 4.0 and 37° for 5 to 60 minutes, but these actions diminish after incubation for 90 to 120 minutes.

4) They are rather resistant to alkali and show no inactivation when kept at pH 9.0 and 37° 17 hrs. When the maceration is kept at pH 11.5 and 37°C, it loses activity of gelatin- as well as benzoyldiglycine-splitting after incubation for 60 minutes and it remains only action of peptone (Riedel)- and slight benzoylglycine-splitting after treatment for 120 minutes. Peptonase and hippurase are more resistant against both treatments than others.

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# ON THE INTERMEDIATE COMPOUND BUILT IN THE PROCESS OF CATALASE REACTION.*

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On investigating the action of various poisonous substances upon catalase activity, we have observed that the degree of inhibition often varied with time showing two distinct phases which we have called "initial" and "final" states of the inhibition. Using both kinetic and spectrophotometric techniques we were able to make it clear that the initial inhibitory state is based on a reaction between the poison and the free form of the catalase molecule, while the final inhibition state is brought about by the reaction of the poison with a certain state of the catalase molecule which is built as an intermediate step in the process of the catalase reaction. Experimental evidence has been adduced which indicates that the intermediate state in question is a complex in which the catalase molecule is combined with $H_2O_2$ molecule in a reversible manner. Based on these findings and inferences we pictured a mechanism of catalase action as being composed of the following three consecutive reactions:

$$E + S \underset{k_1'}{\overset{k_1}{\rightleftharpoons}} \overset{S}{E} \qquad (1)$$

$$\overset{S}{E} + S \underset{k_2'}{\overset{k_2}{\rightleftharpoons}} \overset{S}{SE} \qquad (2)$$

$$\overset{S}{SE} \xrightarrow{k_3} E + 2H_2O + O_2 \qquad (3)$$

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where E is the free catalase molecule, S hydrogen peroxide, $\overset{S}{E}$ the intermediate complex in question, and $S\overset{S}{E}$ a complex in which another molecule of $H_2O_2$ is bound reversibly to $\overset{S}{E}$. It is assumed that the reaction (1) can take place almost instantaneously and that the equilibrium attained is shifted extremely towards the right-hand side. According to these assumptions, it is inferred that on the addition of $H_2O_2$ to a catalase solution practically all existing catalase molecule will take the forms $\overset{S}{E}$ or $S\overset{S}{E}$. At the stationary state the concentration ratio of these two forms will be

$$\frac{k_2'+k^3}{k_2\,[S]} = \frac{[\overset{S}{E}]}{[S\overset{S}{E}]}$$

and the rate ($v$) at which hydrogen peroxide is decomposed will be

$$v = \frac{2k_2k_3\epsilon\,[S]}{k_2'+k_3+k_2\,[S]} \qquad (4)$$

In ordinary experiments of catalase reaction we usually use a rather low concentration (about $10^{-2.0}$ mole/lit.) of hydrogen peroxide, and under such a condition it is known that the rate $v$ is directly proportional to the concentration of hydrogen peroxide. This means $k_2\,[S] \ll k_2'+k_3$ in Eq. (4), so that in ordinary experiments $[\overset{S}{E}] \gg [S\overset{S}{E}]$, *i. e.* practically all existing catalase molecules will assume the form of $\overset{S}{E}$ when a small quantity of $H_2O_2$ is added to the catalase solution.

If a certain poison (G) is added.....in the absence of $H_2O_2$.....to a catalase solution, the following reversible reaction occurs

$$E + G \overset{K_G}{\rightleftharpoons} EG \qquad (5)$$

When $H_2O_2$ is added to a system in which the equilibrium between E and G had been attained, all existing E will be changed into $\overset{S}{E}$, entailing the destruction of the equilibrium (5); the poison G will then commence to react with $\overset{S}{E}$ in the following manner:

$$\overset{S}{E} + G \overset{K'_G}{\rightleftharpoons} \overset{S}{E}G \qquad (6)$$

As we have shown in one of our previous papers (*1*), the degree of inhibition ($H$) defined by

$$H = 1 - \frac{V_G}{V} \qquad (7)$$

($V_G$ and $V$ denoting the apparent velocity constant of $H_2O_2$ decomposition in the presence and absence, respectively, of the given poison), follows, both at the "initial" and the "final" states, the equation:

$$H = \frac{G}{\phi + G} \qquad (8)$$

where $G$ is the concentration of the poison and $\phi$ a constant corresponding to the poison concentration causing 50% inhibition. The $\phi$-values in the initial state is usually larger than that in the final state, so that the degree of inhibition becomes larger as the inhibition proceeds from the initial to the final state. On the assumption described above the $\phi$-values at the initial and final states are nothing but the dissociation constants of EG and $\overset{S}{E}G$, respectively, and the fact that the transition from initial to final state takes place with a measurable velocity indicates that the reaction (6) between $\overset{S}{E}$ and G occurs rather slowly. The only exception to this rule was the behavior of cyanide which showed no distinction between the "initial" and "final" states. In this case it appears that the poison reacts with $\overset{S}{E}$ very rapidly, and the resulting $\overset{S}{E}G$ seems to have the same dissociation constant as EG.

While the series of our studies reported here as well as those reported earlier (*1, 2. 3, 4* and *5*) have been completed without being able to be published in due time in foreign languages, the papers of Chance (*8*) have become available to us, which.....in our opinion.....brought out extremely important data in support of our hypothesis outlined above. This worker has found that on the addition of $H_2O_2$ to a catalase solution the light absorption at 405 m$\mu$ decreases by about 10%. Taking advantage of this fact and using the flow-method originally invented by Hartridge, Roughton and

Millikan, Chance measured the velocity and equilibrium of the formation of $H_2O_2$-catalase complex which he thought, with good reason, to be an intermediate of the catalase reaction. According to our opinion, the complex observed by Chance is nothing but $\overset{S}{E}$ postulated in our theory, which can be shown by the fact that the kinetic data obtained by Chance coincide satisfactorily with quantitative deductions drawn from our theory.

The investigations reported in the present paper deal with three objects: (1) to study in detail the kinetic aspect of the reaction between G and $\overset{S}{E}$ using slow reacting poisons, sodium azide and *o*-chlorophenol, (2) to substantiate our assumption of the intermediate complex $\overset{S}{E}$ using monomethyl hydrogen peroxide as a substitute of hydrogen peroxide, and (3) to interprete the data reported by Chance on the basis of our theory.

Experiments were made, as in the previously reported works (*1* and *6*), with purified equine liver catalase, using methods already described in detail. All experiments were carried out at pH 7.0 (phosphate buffer of 1/150 mole/lit. in final experimental solutions).

### *The velocity of reaction between the intermediate catalase complex* ($\overset{S}{E}$) *and some poisons.*

It is assumed that, owing to the rapidness of reaction (1) and the fact that its equilibrium is shifted extremely toward the right-hand side, practically all catalase molecules will be converted into the form $\overset{S}{E}$ as soon as $H_2O_2$ is added to the catalase solution. If such a poison as azide or phenols is added together with $H_2O_2$ to the catalase solution, the first event to occur will also be the total conversion of existing catalase molecule into $\overset{S}{E}$, which, however gradually, reacts with the poison in the following manner:

$$\overset{S}{E} + G \underset{k_5'}{\overset{k_5}{\rightleftharpoons}} \overset{S}{E}G \qquad (9)$$

As long as the reaction towards opposite direction can be neglected, the velocity of the reaction will be

$$-\frac{d[\overset{S}{E}]}{dt} = k_5 [\overset{S}{E}][G] \qquad (10)$$

Denoting the total concentration of catalase by $\varepsilon$, *viz.*

$$\varepsilon = [\overset{S}{E}] + [\overset{S}{E}G]$$

we have from (10)

$$k_5 = \frac{-\ln [\overset{S}{E}]_t/[\overset{S}{E}]_0}{[G] \cdot t} = \frac{-\ln [\overset{S}{E}]_t/\varepsilon}{[G] \cdot t} \qquad (11)$$

where $k_5$ is the velocity constant and $[\overset{S}{E}]_t$ the concentration of $\overset{S}{E}$ at t seconds after the addition of the poison.

As the reaction (9) proceeds, the rate of $H_2O_2$ decomposition will correspondingly be decreased, since the decomposition rate is proportional to the concentration of $\overset{S}{E}$ at each moment. The degree of inhibition ($H_t$) at the time t, as is defined by Eq (7), can be expressed by

$$H_t = 1 - \frac{[\overset{S}{E}]_t}{\varepsilon} \qquad (12)$$

which will become gradually larger as the reaction (9) proceeds. Substituting this relation into (11), we have

$$k_5 = \frac{-\ln (1-H_t)}{[G] \cdot t} = \text{constant.} \qquad (13)$$

Test was made of this equation by using sodium azide and *o*-chlorophenol as poisons and determining the *H*-values at different moments after addition of the poison. The time courses of $H_2O_2$ decomposition observed in the experiment with azide are illustrated in Fig. 1.

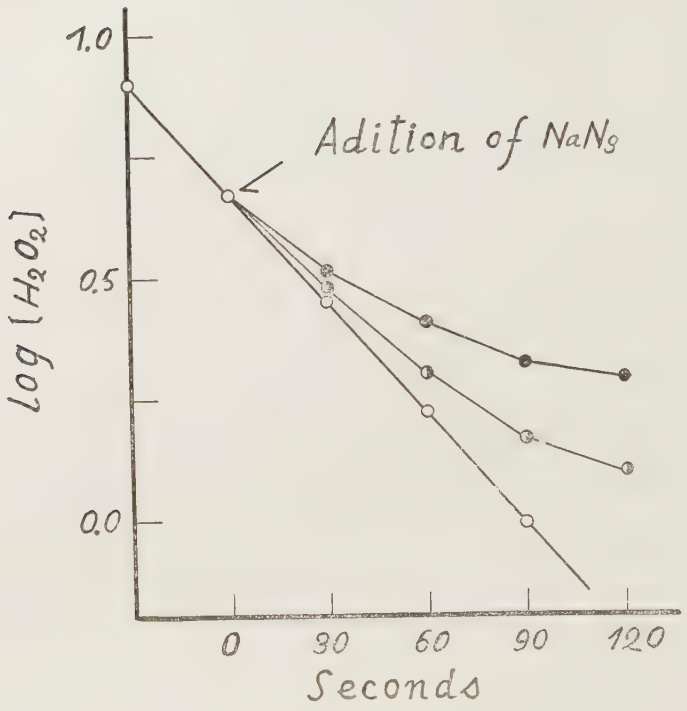


Fig. 1. Characteristic bending of the $\log[H_2O_2]$-t-curve occuring on the addition of $NaN_3$ at pH. 7.0 and 0°.

——○——: control; the $\log[H_2O_2]$-t-curve in absence of $NaN_3$.
——◑——: the $\log[H_2O_2]$-t-curve in presence of $NaN_3$ ($10^{-7.5}$ mole/lit.).
——●——: the $\log[H_2O_2]$-t-curve in presence of $NaN_3$ ($10^{-7.0}$ mole/lit.).

TABLE 1.

*Determination of the velocity constant $k_5$ for azide and o-chlorophenol.*

(at pH 7.0 and 0°).

To a buffered catalase solution (16 ml. of 1/120 mole/lit. phosphat buffer) was added, at first, 2.0 ml. of $H_2O_2$ solution (ca. 0.1 mole/lit.); after 30 seconds, 2.0 ml. of poison solution wss introduced and by titrating the remaining $H_2O_2$ at different times, the gradual increase of the *H*-value was traced.

Azide

| | t (sec.) | 15 | 45 | 75 | 105 |
|---|---|---|---|---|---|
| p [$NaN_3$]* =7.5 | $1-H_t$ | 0.89 | 0.78 | 0.62 | 0.29 |
| | log $k_5$ | 5.36 | 5.23 | 5.32 | 5.37 |
| p [$NaN_3$] =7.0 | $1-H_t$ | 0.72 | 0.50 | 0.36 | 0.16 |
| | log $k_5$ | 5.35 | 5.24 | 5.10 | 5.24 |

average value of $k_5=10^{5.30}$ lit./mole·sec.

*o*-chlorophenol

| | t (sec.) | 45 | 90 | 165 |
|---|---|---|---|---|
| p [HO–C$_6$H$_4$–Cl] =3.9 | $1-H_t$ | 0.17 | 0.31 | 0.55 |
| | log $k_5$ | 1.84 | 1.72 | 1.77 |

average value of $k_5=10^{1.78}$ lit./mole·sec.

In conformity with our expectations, the $H$-values which varied with time gave constant $k_5$-value when applied to Eq. (13). The average values of the constant were as follows:

$k_5=10^{5.30}$ lit./mole·sec. for azide,

$k_5=10^{1.78}$ lit./mole·sec. for *o*-chlorophenol.

To make clear the nature of the reaction in question, further experiments were made on the influence of ionic strength upon the velocity constant $k_5$. Change of ionic strength of the medium was effected by adding varying concentrations of sodium sulfate, which

* p [$Na\ N_3$] = – log [$Na\ N_3$]

itself has been confirmed to be non-injurious upon catalase action at least in a concentration below 0.04 mole/lit. The results obtained were worthy of note; while the reaction between azide or fluoride and E was not influenced at all by the variation of ionic strength, the corresponding reaction of *o*-chlorophenol was decidedly retarded by the increase of ionic strength of the medium. (See illustration

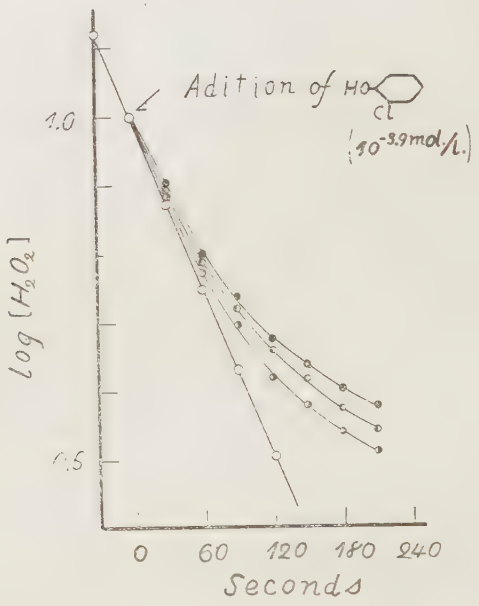


Fig. 2. Bending of the log$[H_2O_2]$-t-curve occuring on the addition of *o*-chlorophenol, and its modification under the effect of ionic strength of the medium: (ionic strength being varied by the addition of $Na_2SO_4$ in different concentrations).

This experiment was carried out at pH. 7.0 and 0°

——○——: control; the log$[H_2O_2]$-t-curve in absence of *o*-chlorophenol and $Na_2SO_4$.

——◑——: the log $[H_2O_2]$-t-curve in presence of *o*-chlorophenol ($10^{-3.9}$ mole/lit.) and in absence of $Na_2SO_4$

——◐——: the log $[H_2O_2]$-t-curve in presence of *o*-chlorophenol ($10^{-3.9}$ mole/lit.) and $Na_2SO_4$ (0.02 mole/lit.)

——●——: the log$[H_2O_2]$-t-curve in presence of *o*-chlorophenol ($10^{-3.9}$ mole/lit.) and $Na_2SO_4$ (0.034 mole/.it.)

in Fig. 2.) These results may be taken as an evidence that the former is a reaction between neutral molecules, while the latter represents a reaction between a cation and an anion (*7*). This conclusion will afford an important clue to the interpretation of various phenomena relating to the pH-dependency of $\phi$-values as will be shown in a later report (*10*).

### *Reversible complex built of monomethyl hydrogen peroxide with catalase, and its significance as a counterpart of the intermediate $H_2O_2$-complex of catalase.*

In one of the previous reports (*1*) we have shown that by previous treatment of catalase with a small quantity of $H_2O_2$, the phenomenon of "initial" inhibition can be totally abolished, the inhibition taking its "final" course from the very beginning of the experiment. Quite similar phenomenon can be brought about by pretreatment of catalase with monomethyl hydrogen peroxide which has a chemical structure analogous to hydrogen peroxide.

An experiment carried out with sodium azide may serve as an example. (See Fig. 3.) A solution (17 ml. in vol.), in which catalase and azide have been allowed to establish their equilibrium $E+G \rightleftharpoons EG$, was supplied with a small quantity (1 ml.) of monomethyl hydrogen peroxide solution (final concentration about $10^{-6.0}$ mole/lit.), and after a lapse of different lengths of time (2, 5, 10, 20, 30 and 40 minutes) 2.0 ml. of $H_2O_2$ solution (final concentration $10^{-2.0}$ mole/lit.) was added and the course of $H_2O_2$ decomposition was followed by titration. As was the case with the pretreatment with $H_2O_2$, the course of the $\log[H_2O_2]$-t-curve is a function of the period of pretreatment (*i. e.* the interval between the addition of monomethyl hydrogen peroxide and the addition of $H_2O_2$ to be decomposed); as the period increased, the grade of inhibition also increased, but when the period exceeded 10 minutes, the $\log[H_2O_2]$-t-curve became linear showing the constant inclination which may be regarded as corresponding to

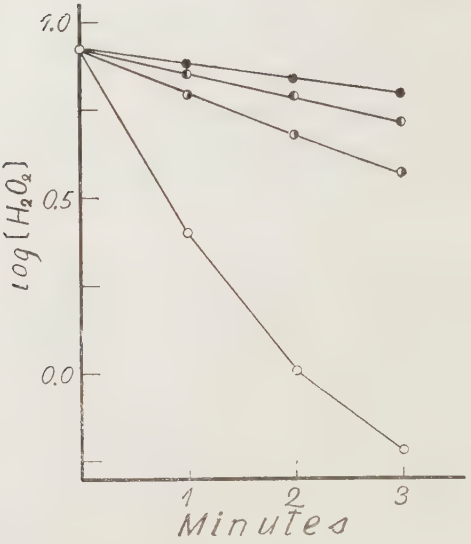


Fig. 3. Effect of pretreatment with monomethyl hydrogen peroxide on the course of $\log[H_2O_2]$-t-curve shown by catalase solutions previously set in equilibrium with $10^{-7.5}$ mole/lit. $NaN_3$ at pH. 7.0 and 0°.

Length of the pretreatment: ——◑—— 2 min; ——◐—— 5 min; ——●——; 10, 20, 30 and 40 min.

——○——: the $\log[H_2O_2]$-t-curve in absence of monomethyl hydrogen peroxide and in presence of $NaN_3$ ($10^{-7.5}$ mole/lit.).

the "final" state observed in ordinary experiments. In the case of pretreatment with $H_2O_2$, this state was attained when the period of pretreatment was about 10 minutes, and when the pretreatment lasted longer, the $\log[H_2O_2]$-t-curve began to deviate from the "final" course, gradually nearing the course which was shown by the solution without pretreatment. This phenomenon was explained as being due to the disappearance of $H_2O_2$ caused by the poison free form of catalase molecule existing in the test solution. The absence of such a phenomenon in the case of pretreatment with monomethyl hydrogen peroxide is obviously due to the fact that the substance is practically inert towards the action of catalase.

That the monomethyl hydrogen peroxide combines with catalase molecule can be demonstrated by the observation of absorption band of the mixture using microspectroscope. Unfortunately, however, the precise nature of this spectrum could not be followed out owing to the fact that the spectrum gradually changed during observation. This change of spectrum was caused by gradual decomposition of monomethyl hydrogen peroxide by the action of the catalase which must have been added in as high a concentration as $10^{-5.0}$ mole/lit. in spectrophotometric experiments. It must be remarked that this decomposition reaction can totally be neglected in kinetic experiments in which the concentration of the catalase applied was always less than $10^{-7.0}$ mole/lit. By using *ca.* $10^{-7.0}$ mole/lit. catalase solution, it was confirmed that the concentration of monomethyl hydrogen peroxide remained constant for more than 30 minutes.

As may be expected, monomethyl hydrogen peroxide itself, owing to its ability to attach itself to the catalase molecule, probably occupying the site to which $H_2O_2$ links, behaves as an inhibitor upon the process of $H_2O_2$ decomposition by catalase. The degree of inhibition increases with the progress of $H_2O_2$-decomposition, a phenomenon similar to that observed with many other inhibitors. At its "initial" state, the inhibition-pG-curve represents a typical sigmoid of the first order, showing the $\phi$-values* of $10^{-5.6}$ mole/lit. and $10^{-5.3}$ mole/lit. at 2.5 and 10 seconds, respectively, after the addition of $H_2O_2$. At the "final" state the curve deviated remarkably, for some reason obscure at present, from the curve of first order sigmoid. (See Fig. 4.) In the present paper, we shall not enter into details of the inhibition caused by monomethyl hydrogen peroxide alone, but restrict ourselves to the description of phenomena which were observed when monomethyl hydrogen peroxide was combined with other poisons whose action mechanism have been studied in detail in our previous works (*1* and *6*).

* These $\phi$-values were estimated at pH 7.0 and 0°.

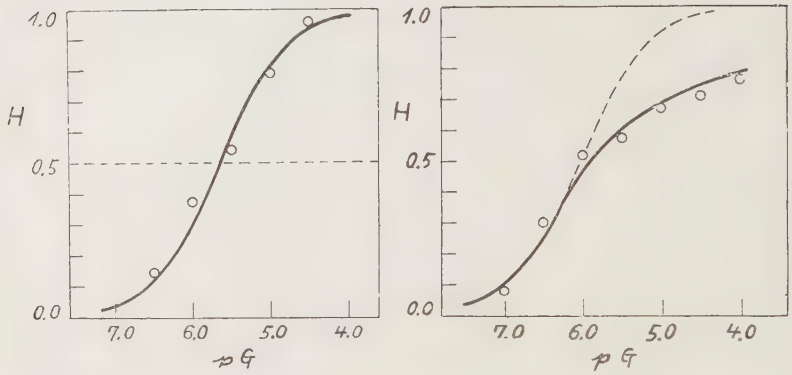


Fig. 4. Inhibition-pG-curves of monomethyl hydrogen peroxide at pH. 7.0 and 0°.

Left; the reaction was stopped at 2.5 seconds after additon of hydrogen peroxide.

Right; the reaction was stopped at 10 seconds after addition of hydrogen peroxide.

The experiments were carried out in the following manner: a catalase solution (9 ml. in volume buffered with 1/135 mole/lit. phosphate to pH 7.0) containing monomethyl hydrogen peroxid (**M**) and a poison (G) to be tested was allowed to attain the thermal equilibrium between **M**, G and catalase at 0°. To this solution 1.0 ml. of $H_2O_2$ solution was added and after 6 seconds, the quantity of $H_2O_2$ decomposed was measured by the method described in our previous papers (*1* and *6*). Varying the concentration of the poison G, while that of **M** was kept constant at $10^{-6.5}$ mole/lit. or $10^{-7.0}$ mole/lit., the degree of inhibition caused by G as a function of its concentration was followed. As in our previous paper (*6*), the degree of inhibition in this case was defined by*

$$H_{1,2} = 1 - \frac{V_{1,2}}{V_1}$$

Where $V_1$ stands for the apparent velocity constant of $H_2O_2$ decomposition in the presence of a definite concentration of **M**, and $V_{1,2}$

*Cf. our previous papers for the meaning of various notations used here.

that in the presence of the same quantity of M and varying concentrations of G. In the concentrations applied, $10^{-6.5}$ mole/lit. and $10^{-7.0}$ mole/lit., monomethyl hydrogen peroxide itself caused only about 20 % and 10% inhibition, respectively. As the poison G, we tested representatives of the three types discussed in our previous paper, namely, cyanide, azide and *o*-chlorophenol. In all cases, the inhibition-pG-curves were found to represent a sigmoid of the first order. From these curves, the values $\phi_{1,2}$ were obtained and these were compared with $\phi_2$ which were shown by the poison in question in the absence of M.

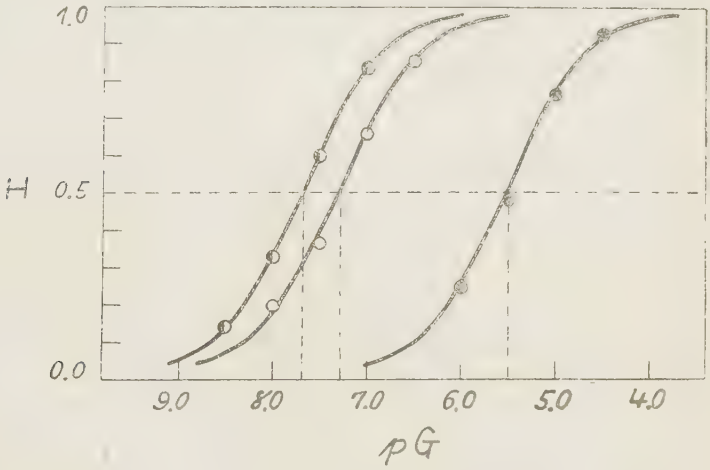


Fig. 5. Inhibition-pG-curves of azide in the presence and absence of monomethyl hydrogen peroxide. (pH. 7.0)

——○——: in the presence of $10^{-7.0}$ mole/lit. monomethyl hydrogen peroxide (0°). Total concentration of catalase used was $\varepsilon = 10^{-7.1}$ mole/lit. $p\phi_{1,2} = 7.3$

——◑——: Final state in the absence of monomethyl hydrogen peroxide (0°) $p\phi' = 7.7$

——●——: Initial state in the absence of monomethyl hydrogen peroxide (7°C) $p\phi = 5.5$.

1) Azide. The $\phi_{1,2}$-value shown by azide in the presence of $10^{-7.0}$ mole/lit. monomethyl hydrogen peroxide was $10^{-7.3}$ mole/lit.

at 0°. In our previous paper (*1*), we had found that the "initial" and "final" $\phi$-values of azide were $10^{-5.5}$ mole/lit. (at 7°.) and $10^{-7.7}$ mole/lit. (at 0°.), respectively. (See illustration in Fig. 5.) The fact that the $\phi_{\frac{1}{2}}$-value is of the same order of magnitude as the "final" $\phi'$-value, both being remarkably smaller than the "initial" $\phi$-value, may be regarded as a strong evidence that, by the influence of monomethyl hydrogen peroxide, the catalase molecule undergoes a certain change which is quite analogous to that caused by the influence of $H_2O_2$.

2) *o*-Chlorophenol. With this poison the $\phi_{\frac{1}{2}}$-value in the presence of $10^{-7.0}$ mole/lit. monomethyl hydrogen peroxide was found to be $10^{-4.8}$ mole/lit. at 0°., a value which is again comparable with the "final" $\phi'$-value of $10^{-5.2}$ mole/lit. at 0°., but a good

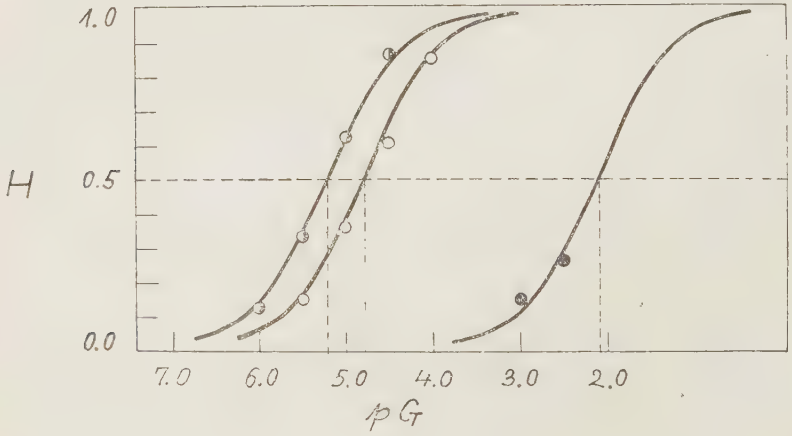


Fig. 6. Inhibition-pG-curves of *o*-chlorophenol in the presence and absence of monomethyl hydrogen peroxide (pH 7.0).

——○——: in the presence of $10^{-7.0}$ mole/lit. monomethyl hydrogen peroxide (0°) $p\phi_{\frac{1}{2}}=4.8$

——◑——: Final state in the absence of monomethyl hydrogen peroxide (0°). $p'\phi = 5.2$

——●——: Initial state in the absence of monomethyl hydrogen peroxide (7°). $p\phi = 2.1$.

deal smaller than the "initial" $\phi$-value of $10^{-2.1}$ mole/lit. at 7°. (See illustration in Fig. 6.)

3) Cyanide. The phenomena observed with cyanide were somewhat different from those described above. The $\phi_{i,2}$-value found in the presence of $10^{-6.5}$ mole/lit. monomethyl hydrogen peroxide was $10^{-6.15}$ mole/lit. at 0°.; this value is the same as the $\phi$-value ($10^{-6.2}$ mole/lit. at 0°.) observed in our previous experiment (*1*). As was emphasized in our earlier report, cyanide has a peculiar property showing no distinction between "initial" and "final" state in its inhibitory action upon catalase. This fact was explained by the assumption that cyanide has the same affinity to E and $\overset{S}{E}$, with which other poisons such as azide and phenols react with different affinities. The fact that the $\phi$-value of cyanide is not modified by the presence of monomethyl hydrogen peroxide seems to provide further evidence for our assumption that the complex built of this substance and the catalase represents a relevant counterpart of the intermediate $H_2O_2$-complex which we have designated by the symbol $\overset{S}{E}$.

### *The interpretation of the data reported by* Chance.

By the elaborate technique of flow-method, Chance (*8*) has ascertained that the primary reaction between $H_2O_2$ and catalase proceeds at a velocity of $10^{7.48}$ lit./mole·sec. and that the reverse of this reaction occurs at a rate of $10^{-1.7}$ 1/sec. The dissociation constant of the complex in question is, therefore, about $10^{-9.2}$ mole/lit. These values were measured at room temperature which, for the present purpose, may be assumed to have been about 20°.

Besides the important data obtained by Chance, there are now available two more quantitative data concerning the kinetics of catalase reaction. In an experiment to be reported later (*10*) we have measured the Michaelis constant of the catalase reaction and obtained the figure: $10^{-0.33}$ mole/lit. at 22°. On the other hand, it

was ascertained by Bonnichsen, Chance and Theorell (*9*), that the overall velocity constant of the catalase reaction is $10^{7.51}$ lit./mole·sec. at 22°.

Based on these data, we can now assign concrete figures to the various rate constants of the three consecutive reactions, (1), (2) and (3), which we assumed for the catalase reaction; *viz.*

$$\left.\begin{aligned} &k_1=10^{7.48}\ \text{lit./mole-sec.}\\ &k_1'=10^{-1.7}\ \text{1/sec.}\\ &k_2'/k_2=10^{-0.33}\ \text{mole/lit.}\\ &k_3=10^{6.91}\ \text{1/sec.}\qquad k_2'\gg k_3 \end{aligned}\right\}\ \text{at about 20°C.}$$

The reasoning for deducing these figures is as follows. According to our assumption, the concentrations of $\overset{S}{E}$ and $\overset{S}{SE}$ will change with the following velocities:

$$\frac{d\,[\overset{S}{E}]}{dt}=k_1\,[E]\,[S]+k_2'\,[\overset{S}{SE}]-k_1'\,[\overset{S}{E}]-k_2\,[\overset{S}{E}]\,[S]$$

$$\frac{d\,[\overset{S}{SE}]}{dt}=k_2\,[\overset{S}{E}]\,[S]-(k_2'+k_3)\,[\overset{S}{SE}]$$

At the stationary state $\frac{d\,[\overset{S}{E}]}{dt}=0$ and $\frac{d\,[\overset{S}{SE}]}{dt}=0$, so that

$$[\overset{S}{E}]=\frac{k_1\,[S]\,\varepsilon}{k_1'+(k_1+k_2)\,[S]+\dfrac{k_2\,[S]\,(k_1\,[S]-k_2')}{k_2'+k_3}} \tag{14}$$

$$[\overset{S}{SE}]=\frac{k_1k_2\,[S]^2\,\varepsilon}{(k_1'+k_1\,[S]+k_2\,[S])\,(k_2'+k_3)-k_2k_2'\,[S]+k_1k_2\,[S]^2} \tag{15}$$

Since the velocity of $H_2O_2$-decomposition is

$$v=\frac{-d\,[S]}{dt}=2\,\frac{d\,[O_2]}{dt}=2\,k_3\,[\overset{S}{ES}]$$

we have

$$v=\frac{2\,k_2\,k_3\,\varepsilon\,[S]}{k_2\,[S]+(k_2'+k_3)\left(1+\dfrac{k_1'+k_2\,[S]-k_2k_2'\,[S]/(k_2'+k_3)}{k_1\,[S]}\right)} \tag{16}$$

If $k_1$ is so large as to fulfill the condition

$$k_1\gg\frac{k_1'}{[S]}+k_2-\frac{k_2\,k_2'}{k_2'+k_3} \tag{17}$$

then

$$v = \frac{2\ k_3\ \varepsilon\ [S]}{[S] + \frac{k_2' + k_3}{k_2}} \tag{18}$$

which indicates that the Michaelis constant of the catalase reaction is $\frac{k_2' + k_3}{k_2}$. Assuming that $k_2' \gg k_3$, we have, as is given above, $k_2'/k_2 = 10^{-0.33}$ mole/lit. In ordinary experiment of catalase reaction, the concentration of $H_2O_2$ applied is of the order of $10^{-2.0}$ mole/lit., a value which is by far smaller than the Michaelis constant mentioned above. Under such a condition, Eq. (4) may be abbreviated to

$$v = \frac{2\ k_2\ k_3}{k_2'}\ \varepsilon\ [S]$$

The value $2\ \frac{k_2\ k_3}{k_2'}$ is the overall reaction constant, which has been found to be $10^{7.54}$ lit./mole-sec. Considering the value of $k_2'/k_2$ given above, we have: $k_3 = 10^{6.91}$ 1/sec.

From Eq (14) it follows, that under the condition $[S] \ll 10^{-0.33}$ mole/lit.

$$[\overset{S}{E}] = \varepsilon$$

which means that, at the stationary state all existing catalase molecules are converted into the form of $\overset{S}{E}$. This inference is in good harmony with our assumption that the $H_2O_2$-catalase complex studied by Chance is nothing but $\overset{S}{E}$ postulated in our theory.

Considering the relative magnitude of $k_1$ and $k_1'$, $k_1\ [S]$ will be overwhelmingly larger than $k_1'$ under ordinary experimental conditions. The condition postulated by (17) is, therefore,

$$k_1 \gg k_2 \left(1 - \frac{k_2'}{k_2' + k_3}\right)$$

which will also be fulfilled when, as we have assumed, $k_2'$ is very large compared with $k_3$.

Summary.

(1) In continuation of works previously reported, quantitative studies were made on the transition phenomena occuring in the inhibitory action of various poisons upon catalase reaction. Further evidence was adduced in support of the hypothesis, advanced in the preceding paper, that the "final" state of inhibition is brought about by the reaction of the poison with the catalase-$H_2O_2$-complex which is formed in an intermediate step of the catalase reaction.

(2) Using azide and *o*-chlorophenol, the velocity constant of the reaction between the catalase-$H_2O_2$-complex in question and the poisons was determined. From the data on the effect of ionic strength of the medium upon the velocity of the said reaction, it was concluded that the reaction between the complex in question and azide is non-ionic, while the reaction between the complex and *o*-chlorophenol is ionic.

(3) By the pretreatment-technique, as was effected with $H_2O_2$ in our previous work, it was shown that a compound quite homologous to the catalase-$H_2O_2$-complex mentioned above is formed between catalase and monomethyl hydrogen peroxide. This compound was found to combine with various poisons with the affinities which are of the same order of magnitude as those shown by the catalase-$H_2O_2$-complex towards the respective poisons.

(4) It was discussed that the catalase-$H_2O_2$-complex, the formation of which was studied kinetically by Chance using the technique of flow-method, is nothing but the complex postulated in the authors' theory. Taking duly into account the data reported by Chance (*8*) and by Bonnichsen, Chance and Theorell (*9*) and also by using some data obtained in the authors' experiment to be reported later, concrete values of velocity constants were assigned to each intermediate step of catalase reaction assumed in the authors' theory. It was thus shown that the kinetic schema:

$$E + S \rightleftarrows \overset{S}{E}$$

$$\overset{S}{E} + S \rightleftharpoons \overset{S}{SE}$$

$$\overset{S}{SE} \rightarrow E + 2H_2O + O_2$$

(E: free catalase molecule, S: hydrogen peroxide, $\overset{S}{E}$: the intermediate complex, $\overset{S}{SE}$: a complex in which another molecule of $H_2O_2$ is bound reversibly to $\overset{S}{E}$), represents the simplest and satisfactory picture in alignment with all kinetic evidences known for the process of $H_2O_2$ decomposition by catalase.

The authors desire, in this opportunity, to thank Dr. H. TAMIYA for his encouragement and support in carrying out this research. Thanks are also due to the Ministry of Education for a grant which enabled the research to be carried out.

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# AMYLASE VALUE IN THE ORGANS AND TISSUES OF VARIOUS ANIMALS.

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Numerous literatures were publicated in both experimental and clinical aspects in relation to the amylolytic activity of the body fluids in this country as well as abroad (references omitted). However, it has called our attention, that very little work has been done in the experimental studies of the amylolytic activity in organs and tissues. It is assumed that this is because the method of amylase determination in organs and tissues heretofore reported, thought to be either lacking in completeness or faced difficulty either in its procedure or theoretical reason.

In determining amylolytic activity of organs and tissues, the following methods have been reported and these methods are same as in the case of blood.

(a) Indirect method: Determination by products of decomposition formed by enzymatic action on substrate, that determines the reductive substances. (1), (2), (3), (4), (5).

(b) Direct method: Determination by quantitative change of substrate itself occured in digestion. (6), (7), (8).

In Method, (A) beside accompanying disadvantage just as in the case of urine or blood, a particular attention has to be paid to the reductive substances other than sugars, which are present much in their own organs and tissues.

But, method (B) is also unsatisfactory in its sensitivity or accuracy, whether it is the method to determine digested starch or the

nephelometric method to evaluate digested glycogen. Since these methods already reported accompany more or less disadvantages in their theoretical reason as well as in practice in the quantitative determination of amylase in organs and tissues, Nakahira has devised a new method of determining amylase value in organs and tissues. In his method he used glycogen as a substrate, and as to evaluate quantitative change of substrate glycogen digested by amylase in the organs and tissues, he applied organs and tissues glycogen determination method orginated by Yamamoto, and adopted results obtained by basic and enzymological preliminary experiments.

It is well known, how amylase value of body fiuid is applicable for practice, and for clinical diagnosis and prognosis. At the same time, experimental studies demonstrated the changes of amylase value of body fluid in all types of metabolic abnormalities. Nevertheless, as far as to amylolytic activity of organs and tissues, very little study has been done and the results thus far obtained have been based on the methods heretofore in use which appeared to be far from completeness. Moreover, in order to ascertain the sources in the changes of amylase value of body fluid, it is necessary for us to know fully the relation between changes in amylase value of organs and tissues and chose in amylase value of body fluid, of animals in normal and abnormal metabolic conditions. To find out changes in amylase value of organs and tissues, it also necessitates to get accurate data as to amylase value of organs and tissues in normal condition of various animals.

Thefefore, the author used the method devised by Nakahira in determination of diastase value of the organs and tissues of various animals and simultaenously determined the amounts of glycogen in organs and tissues by Yamamoto's method, thus attempted to clarify the next basic problem related to amylolytic activity of enzyme.

(1) What are the differences among amylolytic activity of organs and tissues in the same animal?

(2) What are the differences among amylolytic activities of various

animals in the same organs and tissues?

(3) What relation are there between amylolytic activity and quantitative glycogen value in organs and tissues?

*Results of Experiments*

Diastase value in organs and tissues of various animals, namely, rabbits, rats, guinea pigs, bull frogs, chicken, and pigeons were determined. The results are shown in Table I.

The following results were obtained according to Table I.

1. In general, diastase value was highest in kidneys and then in the order of livers, lungs and the lower figure were shown in skins, muscles, and brains.
2. In chicken, amylolytic ferment could not be demonstrated at all in skins and brains.
3. Of animals, generally speaking, diastase value in organs and tissues were highest in rats and guinea pigs.
4. In animals, glycogen content was highest in livers and showed relative increase in diastase value as well, but not the highest.
5. In muscles, diastase value was lower despite of higher glycogen content in general, while in kidneys, diastase vlaue pointed higher in spite of extremely low glycogen figure.
6. It appeared that not much relation existed between glycogen content and diastase value of organs and tissues except the liver. Hence, relation between diastase value and free sugar or hydrolyzable sugar or total sugar contents which closely followed quantitative relation with glycogen content, seemed to be rather slight.

*Summary*

1. Diastase values in the organs and tissues of rabbits, rats, guinea pigs, bull frogs, frogs, chicken and pigeons were determined by the method of Nakahira. Also, glycogen quantities in the organs and tissues were determined by Yamamoto's method at the same time.

2. Among animals, rats and guinea pigs indicated highest diastase values in their organs and tissues.
3. Diastase values were higher in the order of kidneys, livers and lungs in various organs and tissues, while, lower in skins, muscles and brains.
4. In chicken, no demonstrable amylolytic enzyme was found in skins and brains.
5. It appeared that very little relation existed between the diastase value and glycogen of respective organs and tissues.

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| | | Liver | Muscle | Lung | Heart | Skin | Kidney | Spleen | Pancreas | Brain |
|---|---|---|---|---|---|---|---|---|---|---|
| rabbit | diastase value | 1296 | 937 | 553 | 980 | 1191 | 1263 | 794 | 600 | 1010 |
| | glycogen (mg.%) | 2590 | 600 | 126 | 450 | 100 | 140 | 180 | 580 | 266 |
| | hydrolyzable sugar ( „ ) | 410 | 200 | 144 | 300 | 190 | 503 | 260 | 160 | 401 |
| | free sugar ( „ ) | 996 | 169 | 230 | 100 | 234 | 200 | 120 | 200 | 200 |
| | total sugar ( „ ) | 3996 | 969 | 600 | 850 | 524 | 643 | 560 | 940 | 867 |
| rat | diastase value | 2207 | 1969 | 2630 | 1172 | 957 | 3680 | 2194 | 313 | 143 |
| | glycogen ( „ ) | 8814 | 226 | 106 | 75 | 112 | 105 | 120 | 101 | 410 |
| | hydrolyzable sugar ( „ ) | 989 | 295 | 180 | 210 | 200 | 740 | 484 | 200 | 322 |
| | free sugar ( „ ) | 1071 | 111 | 284 | 167 | 210 | 211 | 310 | 206 | 280 |
| | total sugar ( „ ) | 9803 | 632 | 570 | 452 | 500 | 951 | 794 | 507 | 1032 |
| guinea pig | diastase value | 4381 | 3238 | 4289 | 3455 | 606 | 4004 | 4038 | 338 | 839 |
| | glycogen ( „ ) | 910 | 632 | 166 | 420 | 58 | 87 | 217 | 94 | 287 |
| | hydrolyzable sugar ( „ ) | 726 | 300 | 366 | 284 | 252 | 313 | 377 | 706 | 439 |
| | free sugar ( „ ) | 834 | 496 | 234 | 116 | 110 | 185 | 180 | 152 | 274 |
| | total sugar ( „ ) | 2500 | 1428 | 766 | 820 | 420 | 585 | 774 | 952 | 1000 |
| chicken | diastase value | 390 | 263 | 2650 | 340 | 0 | 2250 | 355 | 240 | 0 |
| | glycogen ( „ ) | 1000 | 484 | — | 84 | 90 | 66 | 90 | 56 | 332 |
| | hydrolyzable sugar ( „ ) | 470 | 132 | — | 201 | 288 | 336 | 110 | 200 | 334 |
| | free sugar ( „ ) | 1962 | 200 | — | 330 | 170 | 206 | 200 | 200 | 134 |
| | total sugar ( „ ) | 3432 | 766 | — | 515 | 558 | 508 | 400 | 456 | 800 |

| | | Liver | Muscle | Lung | Heart | Skin | Kidney | Spleen | Pancreas | Brain |
|---|---|---|---|---|---|---|---|---|---|---|
| pigeon | diastase value | 558 | 239 | 2334 | 261 | 295 | 240 | 412 | 440 | 123 |
| | glycogen ( „ ) | 2904 | 1010 | 370 | 70 | 82 | 1060 | 110 | 525 | 390 |
| | hydrolyzable sugar ( „ ) | 394 | 320 | 300 | 190 | 198 | 320 | 140 | 700 | 210 |
| | free sugar ( „ ) | 1622 | 202 | 130 | 150 | 200 | 160 | 210 | 210 | 50 |
| | total sugar ( „ ) | 4920 | 1532 | 800 | 410 | 480 | 140 | 460 | 1335 | 650 |
| bull frog | diastase value | 183 | 839 | 1263 | 400 | 714 | — | 400 | 440 | 300 |
| | glycogen ( „ ) | 8000 | 500 | 315 | 297 | 98 | — | 200 | 525 | 200 |
| | hydrolyzable sugar ( „ ) | 840 | 140 | 210 | 200 | 328 | — | 200 | 700 | 180 |
| | free sugar ( „ ) | 720 | 160 | 150 | 120 | 222 | — | 180 | 210 | 100 |
| | total sugar ( „ ) | 9560 | 800 | 675 | 597 | 648 | — | 580 | 1335 | 380 |
| frog | diastase value | 1630 | 900 | — | — | 960 | — | — | — | — |
| | glycogen ( „ ) | 410 | 200 | — | — | 211 | — | — | — | — |
| | hydrolyzable sugar ( „ ) | 400 | 360 | — | — | 296 | — | — | — | — |
| | free sugar ( „ ) | 690 | 200 | — | — | 266 | — | — | — | — |
| | total sugar ( „ ) | 1500 | 760 | — | — | 746 | — | — | — | — |

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# BLOOD LACTATE AND PYRUVATE OF ALLOXAN-DIABETIC RABBITS.

By

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The physiological action of insulin was investigated by many workers, but still it is not sufficiently clear what stage of carbohydrate metabolism is the chief point of insulin action. Since in 1943 alloxan diabetes was discovered by Dunn, Sheehan, and McLetchie (1), Brunshwig, Allen, Goldner, and Gomori (2), Bailey and Bailey (3), investigations on the experimental diabetes have shown rapid progress. Price, Cori, and Colowick (1945) (4) found that the hexokinase activity of rabbit tissues was inhibited by anterior pituitary extracts, while this inhibition was counteracted by insulin, and thus insulin accelerated the production of glucose-6-phosphoric acid from glucose under the existence of adenosine triphosphoric acid. Broh-Kahn and Mirsky (1947) (5) repeated the experiments of Price, Cori and Colowick, and stated that such results were not always observed, and also that the inhibition of hexokinase was brought about by some extract of spleen, too. Later, Colowick, Cori, and Slein (1947) (6) reported that adrenal extracts also inhibited the action of muscle hexokinase, and insulin counteracted this action. But Stadie and Haugaard (1949) (7) could not find any decrease of hexokinase activity in the muscles and livers of alloxan-diabetic rabbits.

Krahl and Cori (1947) (8) found the decreased glucose utilization of isolated diaphragms of alloxan-diabetic rabbits and the utilization was recovered by adrenalectomy or the addition of insulin

to the test fluid. The diaphragms of hypophysectomized rabbits showed the improved glucose utilization and this was further improved by the addition of insulin (9). Improved glucose utilization and glycogen formation of isolated diaphragms by the addition of insulin were also observed by Gemmill *et al.* (1939, 1941) (10) and Stadie, Zapp (1947) (11), but Perlmutter and Greep (1949) (12) could not find any differences between the diaphragms of hypophysectomized and normal animals.

Thus the effect of insulin on the glucose utilization in the animal body is a noteworthy problem in the light of newer investigation. The author wished to contribute to the solution of this problem, by examining the blood glucose, lactate, and pyruvate in the normal and alloxan-diabetic rabbits under various conditions. The results obtained are described below.

## Experimental

### *Methods*

Male or female rabbits, weighing about 2 kilograms, were used, which were kept on the laboratory diet for several days before the experiments. To determine the blood constituents, the rabbits were always not fed for 16 to 18 hours before the experiments. After the rabbits were fixed as calmly as possible, they were kept at the fixed position at least for 25 minutes, in order to eliminate the effects of stimulation. The blood samples were withdrawn from the rabbits by heart puncture.

To make the rabbits diabetic, a 5 per cent solution of alloxan monohydrate in saline was injected intravenously in a dose of 200 mg. per kilogram of body weight. When the hypoglycemic convulsions took place following the injection, about 1-2 ml. of 20 per cent solution of glucose were injected intravenously each time.

To observe the effects of insulin, glucose, or glucose plus insulin, the following doses were injected intravenously: (1) insulin (Miniglin), 0.8 units per kilogram of body weight, (2) glucose, 2.0

g., dissolved into 10–12 ml. of water, per kilogram of body weight, (3) the same doses of insulin and glucose as above, mixed. The blood samples were obtained from the rabbits before and 15, 30, 60, 120, 150, 300 and 420 minutes after the injection.

Blood sugar was determined by the modification of Fujita-Iwatake's of Hagedorn-Jensen's method (13), blood pyruvate by the Shimidzu's modification (14) of Friedemann-Haugen's method (15), and blood lactate by Barker-Summerson's method (16) with Pulfrich's Stufenphotometer.

*Results*

*1) Blood sugar, lactate, and pyruvate of alloxan-diabetic rabbits*— Blood sugar, lactate, and pyruvate values of alloxan-diabetic rabbits were compared with those of normal rabbits. The average values of 38 normal rabbits and 25 alloxan-diabetic rabbits are shown in Table I. In spite of the remarkable increase of the blood sugar in the diabetic, the blood lactate and pyruvate never increased, so that

TABLE I.

| Conc. in Blood (mg/dl) | max. | min. | average |
|---|---|---|---|
| | Normal Rabbits (38) | | |
| Sugar | 172.8 | 88.8 | 112.0 ± 3.62 |
| Lactate | 177.0 | 21.5 | 67.6 ± 5.94 |
| Pyruvate | 6.16 | 0.76 | 2.91 ± 0.202 |
| S : L | 6.64 | 0.68 | 2.31 ± 0.238 |
| S : P | 126.6 | 19.47 | 46.51 ± 3.605 |
| L : P | 80.95 | 5.98 | 27.58 ± 2.850 |
| | Diabetic Rabbits (25) | | |
| Sugar | 488.0 | 200.3 | 329.9 ± 15.19 |
| Lactate | 210.0 | 21.8 | 63.2 ± 7.73 |
| Pyruvate | 3.85 | 1.05 | 2.24 ± 0.619 |
| S : L | 15.79 | 1.36 | 6.76 ± 0.689 |
| S : P | 255.5 | 61.20 | 174.6 ± 19.12 |
| L : P | 59.66 | 7.33 | 29.29 ± 2.639 |

the ratio of lactate or pyruvate to sugar decreased remarkably. There were no differences of the ratio of lactate to pyruvate between the normal and alloxan-diabetic rabbits.

2) *The effects of insulin injection*—Six normal and four alloxan-diabetic rabbits were used to observe the effect of insulin injection on the blood glucose, lactate and pyruvate. In the normal rabbits the blood sugar began to decrease after the injection of insulin, and after 30 to 60 minutes the minimum value was attained, that was about one-half of the initial value. Thereafter the values began to increase and reached the normal value in about 180 minutes. Blood lactate and pyruvate decreased also, but the minimum values were attained after 2 to 3 hours (Fig. 1 (a)).

When alloxan-diabetic rabbits were injected with insulin, the blood sugar did not decrease so rapidly as in the normal rabbits, but the minimum value was attained after 3 hours and thereafter the slow increase was observed, but the original value was not yet attained after 7 hours. Blood lactate and pyruvate showed a slight decrease at first, but they began to increase after 60 minutes and the maximum values were attained after 2 to 3 hours. The degree of increase of pyruvate was smaller than that of lactate (Fig. 1 (b)).

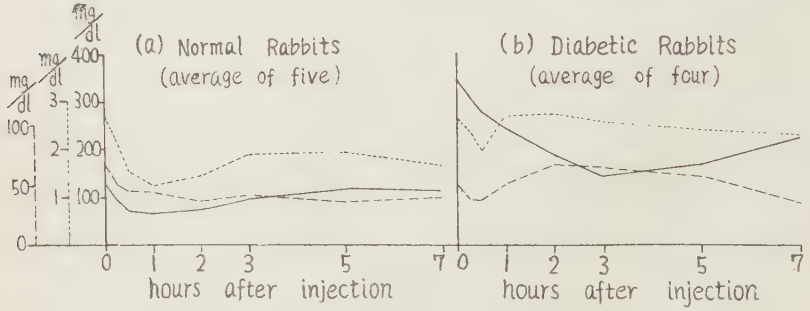


Fig. 1. The effects of insulin injection.
(——blood sugar,— — —blood lactate, ·····blood pyruvate)

3) *The effects of glucose injection*—Eight normal and five diabetic rabbits were used to observe the effects of glucose injection on

the blood glucose, lactate, and pyruvate. In the normal rabbits the increased values of blood sugar began to diminish soon after the injection and 2 or 3 hours later normal values were reached. Blood pyruvate and lactate took the similar course. Both of them increased immediately and afterwards decreased gradually. Pyruvate reached to the normal value or subnormal value after 2 to 5 hours, and lactate reached to the normal value after 30 minutes and thereafter to the subnormal value sometimes (Fig. 2 (a)). When diabetic rabbits were injected with glucose, the recovery of glucose was slower than in the normal rabbits and the original value was not attained still after 7 hours. Blood pyruvate and lactate did not show any increase, for they remained almost at normal levels during the whole course of the observation (Fig. 2 (b)).

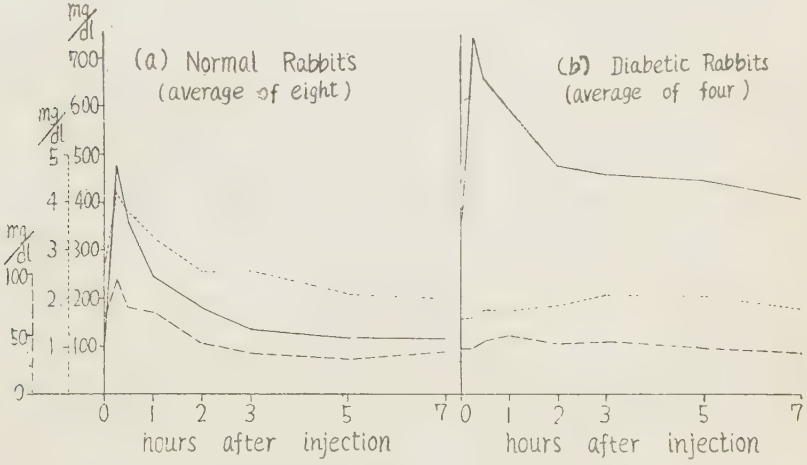


Fig. 2. The effects of glucose injection.
(——blood sugar,— — —blood lactate,·····blood pyruvate)

*4) The effects of simultaneous injection of glucose and insulin*— Seven normal and four diabetic rabbits were used to observe the effects of the simultaneous injection of glucose and insulin. Blood sugar, which was as high as 400 mg. per 100 ml. immediately after

the injection, began to decrease rapidly and after an hour it reached almost to the normal value and after 2 hours to a subnormal value. A slight increase was observed after 5 hours, but the normal value was attained after 7 hours. Blood lactate and pyruvate took the similar course and they showed the maximum values immediately after the injection or 30 minutes later. However, both of them, especially pyruvate, increased frequently after 3 hours (Fig. 3(a)). With alloxan-diabetic rabbits blood glucose, which was at its maximum immediately after the injection, decreased gradually and reached the original value after 2 hours and it still continued to decrease, and the minimum value was attained after 5 to 7 hours. Blood lactate began to increase gradually after the injection, the maximum value was found after 3 to 5 hours, and the normal value was obtained after 7 hours. Blood pyruvate showed the similar increase and it reached the highest value after 1 to 2 hours (Fig. 3 (b)).

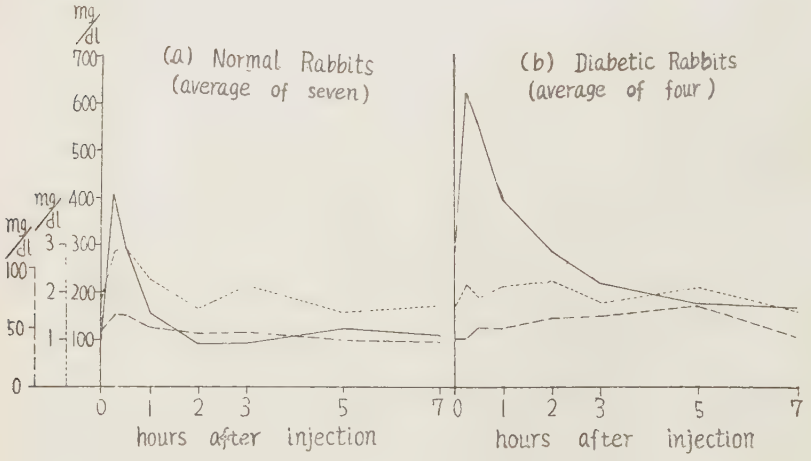


Fig. 3. The effects of glucose plus insulin injection.
(——blood sugar,— — —blood lactate,·····blood pyruvate)

5) *The effects of pyruvate injection*—To observe the rate of

disappearance of the injected pyruvate from the circulating blood, a 5 per cent solution of sodium pyruvate in physiological saline was injected intravenously within 2 minutes in a dose of 150 mg. (calculated as pyruvic acid) per kilogram of body weight. Blood samples were taken before and immediately after the injection and then after 2, 5, 10, 30, 60, and occasionally 120 minutes.

TABLE II.

*Effects of Pyruvate Injection on Blood Glucose, Lactate and Pyruvate Levels of Normal and Diabetic Rabbits.*

| Conc. in Blood (mg/dl) | | before | immediately after | 2 | after (min.) 5 | 10 | 30 | 60 |
|---|---|---|---|---|---|---|---|---|
| | | (a) Normal | | | | | | |
| | Glucose | 122.8 | | | 121.8 | 122.8 | 148.0 | 123.5 |
| | Lactate | 88.8 | | | 124.4 | 128.4 | 127.6 | 150.0 |
| Rabbit | Pyruvate | 3.54 | | | 24.66 | 8.06 | 3.55 | 3.41 |
| no. 363 | | (b) Diabetic | | | | | | |
| | Glucose | 493.0 | | | 402.2 | 443.0 | 424.8 | 409.0 |
| | Lactate | 100.8 | | | 126.3 | 120.0 | 130.0 | 174.3 |
| | Pyruvate | 2.77 | | | 9.42 | 5.78 | 3.95 | 3.93 |
| | | (a) Normal | | | | | | |
| | Glucose | 169.2 | 154.4 | 152.0 | 148.6 | 154.4 | 169.8 | 157.6 |
| | Lactate | 133.0 | 143.8 | 156.0 | 142.2 | 143.7 | 81.0 | 81.0 |
| Rabbit | Pyruvate | 6.16 | 75.36 | 18.76 | 12.02 | 8.43 | 5.95 | 5.63 |
| no. 364 | | (b) Diabetic | | | | | | |
| | Glucose | 456.5 | 421.5 | 416.3 | 415.8 | 450.0 | 409.0 | 428.0 |
| | Lactate | 108.0 | 124.8 | 152.8 | 153.6 | 139.6 | 139.6 | 141.1 |
| | Pyruvate | 1.81 | 68.40 | 22 22 | 8.28 | 8.12 | 3.00 | 2.76 |
| | | (a) Normal | | | | | | |
| | Glucose | 110.5 | 120.4 | 112.6 | 120.2 | 125.5 | 155.0 | 182.3 |
| | Lactate | 68.4 | 68.5 | 63.8 | 60.8 | 51.8 | 52.2 | 44.0 |
| Rabbit | Pyruvate | 3.91 | 58.02 | 8.93 | 4.57 | 4.50 | 4.00 | 3.65 |
| no. 373 | | (b) Diabetic | | | | | | |
| | Glucose | 235.8 | 276.4 | 234.1 | 230.7 | 235.8 | 242.2 | 272.7 |
| | Lactate | 38.5 | 41.5 | 37.6 | 37.6 | 33.6 | 33.4 | 27.5 |
| | Pyruvate | 3.85 | 69.30 | 12.12 | 5.73 | 4.12 | 2.94 | 2.65 |

The course of the change of blood pyruvate, lactate and sugar values were observed with four normal and three diabetic rabbits. In both groups of rabbits blood pyruvate increased remarkably at once but it began to decrease rapidly. The rate of decrease was sometimes a little smaller with the diabetic rabbits, but no essential differences could be found between the two groups (Table II).

6) *The effects of alloxan injection*—In order to observe the course of variations of blood sugar, lactate, and pyruvate values after the injection of alloxan, the following experiments were undertaken. A 5 per cent solution of alloxan monohydrate was injected into normal rabbits in the dose above mentioned, and the blood samples were taken before the injection and after 1, 2, 4, 6, 10, and 24 hours.

The results of two representative cases are shown in Fig. 4. Blood sugar began to increase after the injection and reached its maximum after 1 to 2 hours. Then it decreased rapidly and hypoglycemic state continued for about 4 to 10 hours, and then hyperglycemia took place permanently. Glucosuria was found on the next day. The observation of blood lactate and pyruvate showed

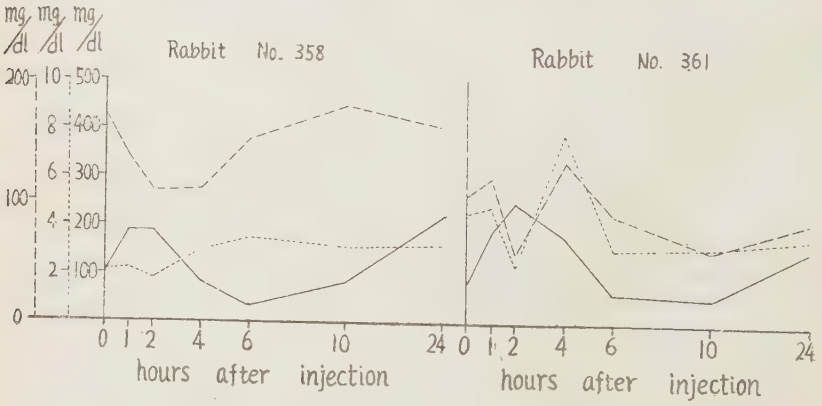


Fig. 4. The effects of alloxan injection on the normal rabbits.
(——blood sugar,— — —blood lactate,……blood pyruvate)

that they did not decrease, or rather increased in the hypoglycemic state and decreased to normal values on the next day.

## Discussion

We have already ascertained the fact that the specific injury of Langerhans' islets was found in alloxan-diabetes (17), as many investigators had stated. In the present report it was found that the concentration of blood sugar is elevated remarkably, but the values of blood lactate and pyruvate are of normal levels in the alloxan-diabetic rabbits. This fact was also observed in the patients of diabetes mellitus or depancreatized animals by us as well as by many other investigators (18–21). If glucose is injected into normal rabbits, the blood lactate and pyruvate are increased in the hyperglycemic phase, but in alloxan-diabetes these are not increased, even if glucose is injected. From these observations it can be concluded that there is some injury of the conversion process from glucose to pyruvate or lactate in the diabetic organism. In fact the elevation of pyruvate or lactate can be observed when insulin is injected into diabetic animals. Thus, insulin is considered as an active agent, which brings about the process of conversion from glucose to lactate or pyruvate.

As above mentioned, hexokinase activity is reported to be inhibited by anterior pituitary, spleen, or adrenal extracts and this inhibition is counteracted by insulin (4–6, 9). Some investigators (7, 12) do not always agree to these observations, but the cause of this inconsistency may be due to the instability of the inhibiting agents. If insulin has relation with the activity of hexokinase, it can be explained clearly that insulin promotes the glucose utilization, namely, glucose oxidation and glycogen formation. There are also some observations that insulin accelerates the fatty acid formation from glucose (22, 23). Broch and Kramer (1948) (24) stated that this action of insulin comes from the acceleration of fatty

acid formation from pyruvate. Furthermore, some investigators considered that insulin accelerates the synthesis of adenosine triphosphoric acid in animal tissues (25–27). If this is the action of insulin, the acceleration of glucose oxidation, glycogen formation and fatty acid synthesis may be completely explained from this action.

In diabetes it is sometimes reported that the oxidation of pyruvate is retarded (28). From our results of pyruvate injection it is not so clear, though some tendency of retardation in its rate of disappearance from circulating blood was also observed. Markee and Meyer (1949) (29) stated that in diabetic acidosis pyruvate disappearance from blood is retarded and this is counteracted by the injection of cocarboxylase but not of insulin. Anyhow, the distinct acceleration of pyruvate disappearance is never observed in diabetes, and lactate pyruvate ratios are the same as normally, and thus, the absence of the elevation of pyruvate or lactate in diabetes must be considered as the result of the damage to the conversion process from glucose to pyruvate.

As for the direct effect of alloxan injection, many investigators observed that there are three phases, namely, the early hyperglycemic, following hypoglycemic, and lastly, persistent hyperglycemic. Many investigators considered the outflow of too much insulin from the necrotic Langergans' islets as the cause of the early hypoglycemic phase (3, 30, 31), and Dunn and McLetchie (1943) (1, 32) considered that the increased secretion of insulin was caused by the stimulation of alloxan. But Houssay et al. (1945) (33) regarded this hypoglycemia as the result of the decrease of glucose formation of the liver, and Bailey et al. (1949) (34) stated they could not determine which opinion was tenable. Now, if the increased secretion or outflow of insulin, or the decreased formation of glucose in the liver were the only change in this phase, the values of lactate and pyruvate should be decreased in accordance with the hypoglycemia, but the results of the above experiments show that both lactate and pyruvate are not decreased or in some cases rather increased in the

blood during this phase. This appearance is similar to the result of the simultaneous injection of glucose and insulin, and thus, it is conceivable that in this phase there still remains the influence of the early hyperglycemic phase.

## Summary

1) In spite of the remarkable increase of blood sugar in the alloxan-diabetic rabbits, their lactate, pyruvate and the ratio of lactate to pyruvate in the blood were of the same levels as in the normal rabbits.

2) When insulin was injected, a distinct decrease of the blood pyruvate and lactate was observed during the hypoglycemic state in the normal rabbits, whereas a temporary decrease and a following increase of the blood pyruvate and lactate were observed in the alloxan-diabetic rabbits.

3) When glucose was injected, the blood pyruvate and lactate as well as glucose were increased in the normal rabbits, but no increases of the former two were observed in the alloxan-diabetic rabbits.

4) When insulin was injected with glucose, blood lactate and pyruvate showed a distinct increase in both normal and diabetic rabbits, although the course was somewhat different between them.

5) The rate of disappearance of the injected pyruvate from the circulating blood showed no remarkable differences between normal and diabetic rabbits.

6) In the hypoglycemic phase of alloxan injection the blood lactate and pyruvate were never decreased, or in some cases somewhat increased.

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